NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

OCTOBER 1, 1983 TO SEPTEMBER 30, 1984

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1983 - September 30, 1984

INTRAMURAL RESEARCH

Project Reports

Laboratory of Biochemistry

Cardiology Branch

Laboratory of Cellular Metabolism

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Hypertension-Endocrine Branch
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Annual Report

Section on Enzymes, Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1984

A. Role of Oxygen Radical Mediated Inactivation of Enzymes in Protein Turnover, Aging and Neutrophil Action

(a) Mechanism of the Inactivation Reaction. Earlier studies demonstrated that many enzymes are inactivated by mixed-function oxidation (MFO) systems which are widely distributed in biological tissues. Results to date support the view that MFO systems catalyze the formation of H_2O_2 and Fe(II) which react at a metal binding site on the enzyme to generate activated oxygen species which oxidize amino acid residues in the enzyme. With several enzymes, inactivation is accompanied by the loss of a single histidine residue, presumably at a metal binding site (catalytic site) and also with the generation of one or more carbonyl groups on the enzyme. Following fragmentation of "oxidized" glutamine synthetase (GS) with cyanogen bromide, a pentapeptide containing the modified histidine has been identified. Prior to modification, this peptide has the sequence (Met-His*-Cys-His-Met) where His* represents the histidine that is susceptible to oxidative modification. It is now evident that the carbonyl group(s) that result from MFO-catalyzed oxidation of GS is not directly associated with the histidine modification but results from the oxidation of another, as yet unidentified, amino acid residue. It was found that any one of several amino acid homopolymers including poly-L-histidine, poly-L-lysine, poly-L-proline, poly-L-serine yield carbonyl derivatives when oxidized by MFO systems. Therefore, any one of several different amino acid residues or proteins must be considered as targets for oxidative attack.

(b) Aging. Other workers have shown that a number of enzymes accumulate as inactive or less active forms during aging. The possibility that these altered enzymes are generated by MFO-catalyzed oxidation reactions is supported by the finding that with increasing age of human erythrocytes, there is a decrease in specific activities of three marker enzymes (triose-phosphate dehydrogenase, glucose-6-P dehydrogenase and aspartate amino transaminase) and this is paralleled by an increase in the carbonyl content of erythrocyte protein. A relationship between aging and protein oxidation is indicated also by other observations, namely: (1) the level of protein carbonyl derivatives in cultured human fibroblasts increases with age of the donor over the range of 13 to 60 years; (2) the protein in cultured fibroblasts from patients with accelerated aging diseases (progeria and Werner's syndrome) contain higher levels of carbonyl groups than do fibroblasts from age/sex matched normal individuals; (3) the carbonyl content of 3-phosphoglycerate kinase (PGK) isolated from old rats is considerably higher than the PGK isolated from young animals (both enzyme preparations were supplied by M. Rothstein); (4) age-related changes in heat stability of glucose-6-phosphate dehydrogenase (G6PDH) can be simulated by exposure of G6PDH to oxidation by a MFO system. The demonstration that cholineacetyltransferase and acetylcholine esterase are readily inactivated by the NADH-oxidase system suggests the possibility that oxidative inactivation may be responsible for the subnormal levels of these enzymes found in patients with Alzheimer's Disease (type AD/SDAT).

(c) Studies with Neutrophils and Macrophages. Prompted by the consideration that human neutrophils and macrophages exhibit many characteristics of MFO

systems, studies have been carried out to determine if the bactericidal action of these cells might involve the oxidative inactivation of key bacterial enzymes. It was found that pure (GS) as well as endogenous GS in intact E. coli cells are both inactivated when exposed to human neutrophils when they undergo a period of "oxygen burst" induced by the addition of either phorbol ester (PMA) or the chemotactic peptide, fMLP. When activated by PMA neutrophils produce relatively high amounts of H_2O_2 and O_2^-, but when activated by fMLP they produce less H_2O_2 and no detectable O_2^-. This difference is reflected in the fact that superoxide dismutase inhibits the inactivation of GS by PMA-activated neutrophils but not the inactivation provoked by fMLP activated neutrophils.

The possibility that suicidal damage of neutrophils involves the oxidation of critical enzymes is suggested by the demonstration that, following PMA stimulation the levels of lactate dehydrogenase, phosphoglycerate kinase, triose-phosphate dehydrogenase and pyruvate kinase in neutrophils decreased 50-75% compared to unstimulated cells. Concomitantly, there is an increase in the level of protein carbonyl groups.

(d) Protein Turnover. Based on the demonstration that rat liver and bacteria contain proteases that preferentially degrade the "oxidized" form of some enzymes, it is proposed that MFO-catalyzed oxidation of enzymes is a "marking" step in protein turnover. An alkaline protease that preferentially degrades the oxidized forms of E. coli GS and rabbit liver enolase has now been purified to homogeniety. It is a thiol protease of M_r = 270,000 and is composed of 25,000-30,000 M_r subunits. From kinetic measurements of the dependence of proteolytic susceptibility and the catalytic activity of GS on histidine modification and the generation of carbonyl groups, it is concluded that the loss of catalytic activity is associated with the modification of a histidine residue, but the generation of carbonyl groups and the susceptibility to proteolytic degradation lags behind.

A protease that degrades the oxidized form of GS 10-20 times faster than native GS has been isolated to apparent homogeneity from extracts of E. coli. This protease (M_r 70,000-80,000, PI = 7.0-7.4) has a pH optimum of 9.90. It is stimulated 2-4-fold by 50 μM Zn(II) plus 1 mM $MgCl_2$. It is inhibited by thiol reagents and by aprotinin but not by other serine protease inhibitors.

B. Toxicity and Transport of Bilirubin

Previous studies demonstrated that osmotic opening of the blood-brain barrier permits entry of albumin-bound bilirubin into the brain of rats, providing a potential experimental model for kernicterus, which in newborns can be a serious disease. Contrary to expectations from human clinical observations, results of studies with 50 animals show that bilirubin is removed very rapidly from rat brain. Clearance from the rat brain parallels that from the general circulation. Both have a half-time of 1.6 hours. Thus, removal of cerebral bilirubin likely involves transport or back diffusion into the general circulation. The potential for clearance of bilirubin from human neonatal brain should therefore be considered.

C. Regulation of Glutamine Synthetase Levels in E. Coli

GS formation in E. coli is repressed by growth in media containing high levels of ammonia nitrogen and is derepressed by nitrogen limitation. It is now

observed that the repressive effect of high ammonia concentration can be suppressed when lactose is provided as the sole source of carbon or when D-amino acids (D-glutamate, D-threonine, D-lysine) or glycine are also present in the growth media. The effects of D-amino acids is additive; i.e., a mixture of all D-amino acids is more effective than a saturating level of either one alone. Strains containing mutations which interfere with the positive control of the GS structural gene, GlnG (i.e., glnB and glnD) fail to exhibit a positive response to D-amino acids. Whereas growth on lactose or D-glutamate suppresses the repressive actions of high ammonia, the growth on lactose but not on D-glutamate favors the production of highly adenylylated GS. The biochemical basis for these effects is still unknown.

D. Phosphorylation and Dephosphorylation of Protein Tyrosine Residues

Two highly sensitive techniques have been developed for the detection and quantitation of phosphotyrosine residues in proteins. One takes advantage of the stability of the phosphotyrosine ester bond to alkaline digestion; the other involves the use of antibodies that recognize the phosphotyrosine moieties of protein. By means of the first procedure, the levels of protein tyrosine kinase (PTK) and protein phosphotyrosine phosphatase (PTPase) have been measured in extracts of normal and transformed cells. The levels of PTK in extracts of normal retina and malignant retinoblastoma were the same. However, the retinoblastoma PTK was stimulated by a growth factor from the cells while the retina PTK was not. The levels of PTK and PTPase in psoriatic skin was activated by epidermal growth factor but the activity in normal adjacent skin was not. High levels of PTPase were found in all tissues studied but vary significantly depending on the tissue type. The PTPase in Ehrlich Acities Tumor (EAT) cells is strongly inhibited by heat stable endogenous inhibitors. tRNA was identified as one of the more important inhibitors. This suggests the possibility that tRNA may play a role in the phosphotyrosine interconversion and hence in cellular growth and differentiation.

Preliminary studies with a technique using antibodies which bind to protein phosphotyrosine residues, indicate that this technique can detect ≈ 300 fmol of phosphotyrosine in proteins. The method therefore shows great promise for the detection and isolation of physiological substrates of PTK and PTPase.

E. Metabolism of Branched-Chain Amino Acids

A study of the metabolism of the branched-chain acids has revealed a pathway of metabolism of leucine that is catabolic in bacteria and appears to be synthetic in humans. The pathway depends upon the activity of the enzyme, leucine 2,3-aminomutase, an enzyme dependent upon adenosylcobalamin as a cofactor. Other enzymes which function in the pathway are β-leucine transaminase, coenzyme A transferase, and thiolase. The relative carbon flux through this pathway and the pathway which is independent of cobalamin greatly favors the independent pathway in brain, heart, kidney, and liver. In the testis, however, the cobalamin-dependent pathway accounts for over forty percent of the carbon flux. This suggests that the metabolism of leucine may play an important role in this organ. The relationship between enzyme activity and various disease states such as pernicious anemia and inborn errors of metabolism will be examined.

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Annual Report
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
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Purification and structural studies of selenoproteins: Clostridial glycine reductase is an enzyme complex comprised of three protein components: selenoprotein A, a heat stable 12,000 M.W. protein that contains a selenocysteine residue; protein B, a protein of ca. 200,000 M.W. that contains an essential carbonyl group and protein C, ca. 250,000 M. W. Clostridium sticklandii and Clostridium purinolyticum (a purine fermenting organism) were used as enzyme sources. An improved method for isolation of homogeneous protein A from both of these organisms was developed. This method made use of a preparative HPLC ion exchange column that effectively separated the selenoprotein, a corrinoid protein, ferredoxin, a quinone-dependent phosphatase and the seleno-tRNAs in a single step. The ability to recover all of these proteins in highly purified form by a two step process from crude extracts of the bacteria represents an important advance in technology and greatly facilitates detailed studies of these macromolecules. Milligram quantitites of ^{75}Se-labeled selenoprotein A isolated from both of the bacteria were used for structural studies. A tryptic peptide isolated by reverse phase HPLC that contains the selenocysteine residue has been partially sequenced. Both of the cysteine residues also were found in this peptide. the selenocysteine residue occurs in a unique position near the carboxyterminus of the protein as shown by carboxypeptidase Y digestion analyses. Although the selenoprotein A components from the two bacterial sources are identical in their cysteine and selenocysteine contents they exhibit slight differences in primary amino acid compositions and the protein from C. purinolyticum is somewhat more acidic. These differences are reflected in their biological activities: The protein A from C. purinolyticum is much more active with homologous proteins B and C than with heterologous proteins B and C from C. sticklandii. Selenoprotein A from the latter organism was similarly active in both systems. An improved isolation procedure for obtaining proteins B and C in large amounts also was developed. This procedure makes use of a phenyl-Sepharose chromatographic step that effectively separates proteins B and C and appears to stabilize protein C which then is isolated in highly purified form by a gel filtration step followed by an ion exchange step. The availability of significant quantitites of proteins A, B and C for reconstitution of pure glycine reductase complex will enable studies on the mechanism of action of these components and the identity of the phosphate ester intermediate generated in the reaction to be carried out.

Two enzymes from Clostridium kluyveri that contain selenium in the chemical form of selenomethionine were investigated in detail. These enzymes, thiolase and 3-hydroxybutyryl-CoA dehydrogenase, have high methionine contents. Peptide mapping experiments using reverse phase HPLC were performed with the purified enzymes labeled with ^{35}S and ^{75}Se. The results of these studies indicated that selenium was distributed throughout the primary structure of thiolase and 3-hydroxybutyryl-CoA dehydrogenase in a manner that paralled methionine. Analyses of proteolytic digests of these proteins support a non-specific incorporation mechanism for selenium. However, this mechanism appears to be limited to selenomethionine substitution for methionine since no selenium incorporation in the form of selenocysteine was detected even though numerous cysteine

residues are present in both proteins. In these studies the use of antibodies raised against purified thiolase permitted quantitative immunoprecipitation of the enzyme from crude extracts and thus facilitated the systematic analysis of effects of wide variations in sulfur to selenium ratios on incorporation of selenium. When ^{75}Se-labeled selenomethionine was the source of selenium for C. kluyveri the incorporation of selenium was inversely proportional to the level of added methionine. However when labeled selenite was the source of selenium similar (millimolar) levels of methionine failed to decrease the incorporation of selenium in the enzymes. In both cases methionine was actively taken up and metabolized by the cells. Attempts to exaggerate the amount of selenium incorporation by growth of the organism in media containing lowered amounts of sulfur and increased levels of selenite or selenomethionine failed because of failure of the organism to grow under such conditions.

A collaborative project carried out with Dr. Raymond Burk of San Antonio, Texas has as its aim to purify and identify the 75,000 M.W. Selenoprotein from rat liver that is exported to the serum and appears to have a high turnover rate. The ^{75}Se-labeled protein, prepared by Burk's group, is sent to us for development of isolation procedures. High performance liquid chromatographic procedures using preparative DEAE columns in addition to phenyl-Sepharose and pyridoxal phosphate affinity column chromatography yield highly purified material. The selenium in this protein was shown to be selenocysteine.

Investigations on occurrence, structure, function and biosynthesis of seleno-tRNAs in bacterial and mammalian systems: Seleno-tRNAs isolated from C. sticklandii, from Escherichia coli and also from a methane-producing organism were analyzed for amino acid acceptance activites and for selenonucleoside contents. In addition to the already identified selenonucleside, 5-methylaminomethyl-2-selenouridine, found in all three bacterial species two different but related 2-selenouridines were present in the two anaerobic bacteria. An E. coli mutant defective in the synthesis of 5-methylaminomethy-12-selenouridine incorporated the same amount of selenium into tRNA as the parent strain. The selenonucleoside in the mutant was identified as 5-aminomethyl-2-selenouridine. A procedure devised for the synthesis of this selenonucleoside provided authentic reference material for proof of structure of the biological material. An in vitro methylating enzyme system converted the 5-aminomethyl-2-selenouridine to 5-methylaminomethy-12-selenouridine in the intact tRNA molecule. Thus the step missing in this E. coli mutant is the addition of the terminal methyl group at the 5 position of the uridine residue. In wild type E. coli cells grown on selenium supplemented media (1 μM selenite or selenate) a maximum of 60-70 pmol of 5-methylaminomethyl-2-selenouridine per A260 unit was observed or about 4 mol of this selenouridine per 100 mol tRNA. Under these conditions about an equal amount of 5-methylaminomethyl2-thiouridine also was present. The amount of the selenonucleoside did not increase when the selenium conc. in the culture medium was increased or if the sulfur was decreased to levels which were growth inhibitory. In selenium deprived E. coli a stable tRNA species appeared to be present that could be modified with selenium to form 5-methylaminomethyl-2-selenouridine. By labeling the sulfur analog residues with ^{35}S it was demonstrated that synthesis of the selenium nucleoside upon the subsequent addition of a selenium source was accompanied by a loss of ^{35}S from the 5-methylaminomethyl-2-thiouridine residues. These and other studies suggest that the substrate for selenonucleoside synthesis is the corresponding thionucleoside residue in a tRNA. Selenium incorporation in a crude in vitro enzyme system was completely dependent on the addition of ATP. In each case the product was shown to be a 5-methylamino-

methyl-2-selenouridine residue. This enzyme system appears to be amenable to detailed analysis as to nature of the immediate selenium donor, mechanism of the apparent substitution reaction and type of enzyme catalysts involved.

The major selenium-containing tRNA in C. sticklandii is a glutamate accepting species and in this organism this is the major $tRNA^{Glu}$ species present. The selenium, present in a 5-methylaminomethyl-2-selenouridine residue located in the wobble position of the anticodon, is essential for esterification of the tRNA by its cognate amino acid tRNA synthetase. In order to study the codon-anticodon interaction of this seleno-tRNA the glutamate codons GAA and GAG were synthesized. Under near physiological conditions the charged seleno-$tRNA^{Glu}$ interacted almost equally well with the two glutamate codons. In contrast a glutamate tRNA from E. coli that contains 5-methylaminomethyl-2-thiouridine in the wobble position of the anticodon showed a marked preference for GAA over GAG. Deselenization of the C. sticklandii $tRNA^{Glu}$ abolished the codon stimulated ribosome binding activity. In a cell-free protein translation system the seleno-tRNA charged with tritium-labeled glutamate proved to be a very active substrate for incorporation of glutamate into the nascent protein. This incorporation of glutamate by the wheat germ extract was dependent on added RNA message thus indicating its specific utilization in the system.

Preliminary experiments in which DNA fragments from C. sticklandii were ligated to plasmid DNA and used to transform E. coli gave two positive clones that contained sequences that hybridize to seleno-$tRNA^{Glu}$. Sequence analysis of the cloned DNA is in progress and should provide information concerning the primary structure of the unmodified tRNA.

Investigation of various rat tissues for the presence of selenium-modified tRNAs showed that liver contained the highest amount of selenium (ca. 0.6%) in the bulk tRNA. A number of selenium-containing tRNA species A number of selenium-containing tRNA species were separated by HPLC analysis on a DEAE column. Although the selenium in these species was much more labile than the bacterial seleno-tRNAs it was not in the form of esterified amino acids (selenocysteine or selenomethionine) because neither of these amino acids was detected using ultra sensitive methods. The rat liver seleno-tRNAs are more stable than those found in cultured mouse leukemia cells and also are distinguishable chromatographically. Although seleno-tRNAs in mammalian tissues are much less abundant than those studied in C. sticklandii and Methanococcus vannielii and also appear to be present in the form of labile selenonucleosides, their occurrence nevertheless seems to be fairly widespread.

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National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

The research activities of the investigators in the Section on Metabolic Regulation are mainly concerned with the physical and chemical approaches to resolve the mechanisms of enzyme action and its regulation, and to study the antibody-antigen interaction. Currently, the research is concentrated on (1) regulation of enzymic activity by cyclic cascade systems, Ca(II) and Ca(II)-calmodulin complex; (2) overproducing enzymes which are involved in glutamine synthetase cascade by cloning methods; (3) mechanistic studies of enzyme action and activation; (4) immunochemical studies of protein phosphatase; and (5) development of analytical methods to facilitate biochemical studies. Together, these research programs will provide a better understanding of how biochemical processes work in cells.

I. Regulation of Enzymic Activity

A. Regulation of Mg(II)-ATP-dependent Phosphatase by the Type II cAMP-dependent Protein Kinase Regulatory Subunit

The Mg(II)-dependent phosphoprotein phosphatase is a major phosphatase in rabbit skeletal muscle. This enzyme, termed F_cM, where M is the modulator subunit, is inactivated as isolated but can be activated by kinase F_a (also known as glycogen synthase kinase-3) in the presence of Mg(II) and ATP. The activation mechanism involved the phosphorylation of the modulator by F_a, which caused a conformational change in F_c resulting in activation of the phosphatase activity. The catalytic subunit of cAMP-dependent protein kinase can also phosphorylate the modulator, however, it fails to activate the phosphatase. Nevertheless, the regulatory subunit of the type II cAMP-dependent protein kinase inhibits the phosphatase activity. This inhibition reaction can be reversed by the addition of excess catalytic subunit of the cAMP-dependent protein kinase to form the holoenzyme, R_2C_2. Addition of cAMP which causes the dissociation of R_2C_2 restores the inhibition effect. This observation, together with other results, indicate that the regulatory subunit of the type II cAMP-dependent protein kinase inhibits both the activated phosphatase and the activation of the inactive enzyme.

This observed phosphatase inhibition coupled with the kinase action provides a highly sensitive mechanism in response to changes in cAMP concentration for cyclic cascade systems in which both cAMP-dependent protein kinase and Mg(II)-ATP-dependent protein phosphatase are converter enzymes.

B. Glutamine Synthetase Cascade.

1. Construction of a multicopy plasmid vector to cause overproduction of UT-UR. Glutamine synthetase is regulated mainly by a bicyclic cascade, in which uridylyltransferase (UT) and uridylyl-removing enzyme (UR) serve as the converter enzyme for uridylylating and deuridylylating the regulatory protein, P_{II}, respectively. The covalently modified (P_{IID}) and unmodified P_{II} (P_{IIA}) in turn activate the deadenylylation and adenylylation of the adenylyltransferase, respectively. Purification of the UT-UR enzyme, a bifunctional polypeptide, has

shown to be a difficult task due to its low quantity and instability. Therefore, research has been carried out to clone the glnD gene, which encodes the UT-UR enzyme in E. coli. Through subcloning, a 2.8 kb DNA fragment was shown to comprise the glnD gene; its size is estimated to be 2.6 kb based on the molecular weight of UT-UR. The isolated glnD gene was cloned into a plasmid vector carrying the strong, regulatable λ phage promoter pL to achieve a nearly 800-fold overproduction of UT-UR. Induction of this enzyme was optimized by varying the induction temperature and the growth medium. This study also established the detailed restriction map and the transcriptional direction of the glnD gene.

When a strain (YMC10) bearing pglnD15 was grown in limited nitrogen, its UT-UR enzyme level obtained was the highest; this level decreased by 40% under excess nitrogen, and further reduced to only 10% of the highest level when grown in LB medium. To explore the possible transcripitional regulation of the glnD gene, the promoter region of this gene was inserted into a promoter expression vector and its transcriptional control was analyzed by measuring the galactokinase transcribed from the promoter. The result indicates that the glnD gene is metabolically regulated.

2. Construction of adenylyltransferase overproducing strain. In an effort to construct a strain to overproduce adenylyltransferase, a strain which bears the glnE gene (structural gene for adenylyltransferase in E. coli) was subcloned into pBR322 and subsequently into the strong promoter pL vector. As a result, one achieved a 300-400-fold overproduction of adenylyltransferase.

C. Regulation of Yeast Glutamine Synthetase

Glutamine synthetase in yeast responds to excess glutamine through both repression and inactivation. Inactivation results in loss of the biosynthetic activity (converting glutamate to glutamine) but not transferase activity (converting glutamine to γ-glutamyl hydroximate) which is a nonphysiological reaction. In order to elucidate this regulatory mechanism, the active and inactive forms were separated by DEAE chromatography. The earlier peak exhibits a higher ratio of biosynthetic to transferase activity than the later peak. This activity ratio difference between the two peaks becomes more pronounced upon aging of the enzyme, due to continuous loss of biosynthetic activity of the second peak. The enzyme in the first peak has been purified to homogeneity.

D. Regulation of Enzymes Mediated by Ca(II)

1. Regulation of calmodulin-dependent protein phosphatase. The Ca(II)-calmodulin activated phosphoprotein phosphatase (also known as calcineurin) isolated from bovine brain was found to contain 0.2-0.6 equivalent of phosphate and nearly stoichiometric quantity of nondialyzable Fe(III) and Zn(II). This enzyme is activated by Ca(II)-calmodulin complex. Concomitant with this activation the enzyme was found to undergo first-order time-dependent deactivation. The deactivation required both Ca(II) and calmodulin, and the deactivated enzyme displayed little or no catalytic activity. The deactivation process has likely resulted from ligand induced protein conformational change, and it is accelerated by the presence of substrate, p-nitrophenyl phosphate and by inhibitors such as pyrophosphate, di- and triphosphate nucleotides. However, the inactivation process can be reversed by addition of divalent metal ions like Ni(II), Mn(II) or Mg(II), which have been shown previously to activate the enzymic activity. This study indicates that calcineurin is not simply a Ca(II)-calmodulin stimulated

enzyme, it requires at least one additional metal ion for both structural stability and full activity.

2. Identify endogenous brain substrate for Ca(II)-calmodulin-dependent protein phosphatase. Ca(II)-calmodulin-dependent protein phosphatase (calcineurin) found in several tissues is highly concentrated in mammalian brain. In an attempt to identify endogenous brain substrates for this enzyme, kinetic analyses of the dephosphorylation of several well characterized phosphoproteins purified from brain were carried out. The proteins studied were: G-substrate, a substrate for cGMP-dependent protein kinase; DARPP-32, a substrate for cAMP-dependent protein kinase; Protein K.-F., a substrate for a cyclic nucleotide and Ca(II)-independent protein kinase; and synapsin I, a substrate for cAMP-dependent (site I) and a Ca(II)-calmodulin-dependent kinase (site II). Calcineurin dephosphorylated each of these proteins in a Ca(II)-calmodulin-dependent manner. The results show that DARPP-32, G-substrate, and Protein K.-F. are all potential substrates for calcineurin *in vivo*.

3. Purification and characterization of a Ca(II)-inhibited phosphoprotein phosphatase. A Ca(II)-inhibited protein phosphatase has been partially purified from bovine brain. The enzyme required Mg(II) for activity. It catalyzes the dephosphorylation of two phosphorylated sites on synapsin I selectively. Since synapsin I is not a good substrate for the Ca(II)-calmodulin-dependent phosphatase, the results indicate that the Ca(II)-inhibited phosphatase and calcineurin may function *in vivo* in a coordinated manner. In addition to synapsin I, a phosphoprotein from neurofilaments found in the microtubule system also served as a substrate for this phosphatase.

II. Mechanism of Enzyme Action and Activation.

A. Mechanism of Activation of Ca(II)-Calmodulin-dependent Protein Phosphatase by Ni(II)

Activation of the Ca(II)-calmodulin-dependent protein phosphatase by Ni(II) proceeds with a lag time. Analysis of the lag time reveals that it is a first-order process with a rate constant of ~ 2 min^{-1} at a p-nitrophenyl phosphate concentration of 10 mM. Like the deactivation reaction, the presence of substrate accelerates the activation, suggesting the existence of interaction between the catalytic site and metal ion binding site. The Ni(II) is bound to the 60,000 molecular weight A subunit as evidenced by a Trp-fluorescence change observed due to Ni(II) binding to the A subunit.

B. On the Mechanism of Ca(II) Activation of Ca(II)-Calmodulin-dependent Protein Phosphatase

To determine the number of Ca(II) ions involved in the activation of calcineurin by calmodulin and the contribution of Ca(II) bound to the B subunit of calcineurin, experiments similar to those performed with cAMP phosphodiesterase (Huang et al., Proc. Nat. Acad. Sci. U.S.A. 78, 871, 1981) were carried out in the absence and presence of Mg(II) ion. Preliminary results indicate that in the absence of Mg(II), a minimum of 3.5 calmodulin-bound Ca(II) are required for activation, whereas in the presence of Mg(II), only 1.7 calmodulin-bound Ca(II) are needed. This suggests that in the absence of Mg(II), the activation of the phosphatase is similar to the activation of cyclic nucleotide phosphodiesterase, each required fully liganded calmodulin for activation. In the presence of

Mg(II), only 1.7 calmodulin-bound Ca(II) is needed, indicating that Mg(II) may be able to enhance the affinity of the enzyme for calmodulin not saturated with Ca(II).

C. Mechanistic Study of Phosphorylation and Dephosphorylation of Smooth Muscle Myosin.

It is known that the 20,000 dalton light chain of both heads of a smooth muscle myosin or heavy meromyosin molecule must be phosphorylated before the MgATPase activity of either head can be activated by actin. The two heads of HMM appear to be phosphorylated randomly at equal rates, while those of myosin are phosphorylated in a negatively cooperative manner. Studies were carried out to investigate the cause of this difference between HMM and myosin. The results show that if myosin is first phosphorylated at high ionic strength (0.6 M KCl), where it is monomeric, and then assayed for MgATPase in 0.05 M KCl, the data support a model where the two heads are phosphorylated randomly with equal rates. The data for the dephosphorylation of fully phosphorylated myosin, both in a filamentous and monomeric state, are best explained by a model where dephosphorylation of one head is sufficient to deactivate the entire molecule. With monomeric myosin, the dephosphorylation occurs randomly with equal rates, whereas with filamentous myosin the dephosphorylation appears to be negatively cooperative. The correlation between dephosphorylation of HMM and its MgATPase activity is more complex and is consistent with a positively cooperative dephosphorylation mechanism.

The phosphorylation of HMM and monomeric myosin proceeded via a first-order reaction. However, when phosphorylation is carried out in low ionic strength (0.02 M KCl), where myosin is present as filaments, the time course consists of two experimental functions in which the rate of one myosin head is 6-10 times faster than the other which is located on the same molecule. This suggests that when myosin is polymerized into filaments, the two previously indistinguishable heads either become nonequivalent or are subject to head-head interaction leading to a negativley cooperative phosphorylation.

III. Immunochemical Studies

A. Immunoanalysis of Phosphoprotein Phosphatases

Phosphoprotein phosphatases regulate cellular metabolism together with protein kinases by modulating the fractional phosphorylation of the interconvertible enzymes or proteins. To date, a number of phosphatases have been isolated and grouped into several classes, termed type 1 (ATP-Mg(II)-dependent phosphatase), type 2A (e.g., elF-2 phosphatase and smooth muscle phosphatase I), type 2B (calcineurin) and type 2C (Mg(II)-dependent phosphatase). They differ in their sensitivity to inhibitor proteins, divalent metal ions and in their substrate specificities. Definitive characterization of these enzymes has been hampered by difficulties in maintaining their native state throughout purification procedures. To investigate the relationship between different phosphoprotein phosphatases, highly purified phosphatases from amoebae (type 2A), turkey gizzards (type 2A), bovine cardiac (type 2A), bovine brain (type 2B), rabbit reticulocyte (type 2A), and rabbit skeletal muscle (type 1) were tested for antigenic relatedness. For this purpose, two heterologous antibody preparations, one made against Acanthamoeba type 2A phosphatase and one made against brain type 2B phosphatase, were used. Specific subunit cross-reactivity was analyzed by protein blot

analysis. The results showed that the antibody against type 2A cross-reacted with all the type 2 phosphatases tested, but not with the ATP-Mg(II)-dependent phosphatase or the type 1 catalytic subunit. In the case of calcineurin, both subunits reacted with the type 2A antibody. When a polyclonal antibody made against highly purified calcineurin was used, protein blot immunoanalysis showed that it cross-reacted with both cardiac phosphatase 2A and brain calcineurin (both subunits), but not with type 1 phosphatase. The basis for the cross-reactivity between the type 2 phosphatases, which are different with respect to their molecular weight and composition, is not clear.

IV. Development of Analytical Methods

Organic Extraction of Inorganic Phosphate with Isobutanol/Toluene

There exists a number of methods for measuring [^{32}P]P_i release in phosphatase assays. Among them, the most widely used is the organic extraction procedure in which free P_i is extracted as a phosphomolybdate complex into a 1:1 mixture of isobutanol and benzene. However, the use of benzene is quite harmful for laboratory use because of its toxicity and the fact that it is quite volatile. A modified method which empolyed toluene or xylene in place of benzene has been developed. When benzene was substituted by toluene, the method is equally effective. A complete characterization of the new assay shows that it is versatile, sensitive, reproducible, and accurate over a broad range of P_i from less than 1 pmole to larger than 200 nmole.

Annual Report
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins involves contributions from both ligand-protein and protein-protein interactions. Ligand promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to the numerous examples of stabilization and destabilization of protein structures by ligands, metal ions, and other inorganic ions.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer; each subunit (50,000 M_r) contains a catalytic site with two essential divalent cation sites (n_1 and n_2) and a tyrosyl residue that is the site of covalent modification by enzymatically-catalyzed adenylylation-deadenylylation reactions. The 12 identical subunits of enzyme are arranged in 2 superimposed hexagonal rings of about 140 Å in diameter and centers of adjacent subunits are ~ 45 Å apart. Studies of the interactions of divalent cations, substrates, substrate analogs, and inhibitors with glutamine synthetase from E. coli have continued.

Recently we showed that the very tight binding of 2 Mn^{2+}, L-methionine-S-sulfoximine phosphate, and ADP ($K_A' > 10^{12}$ M^{-1}) formed on each subunit of E. coli glutamine synthetase at pH 7 by phosphorylation of the L-glutamate analog by ATP, stabilizes intersubunit bonding domains. Various analogs of ATP that are substituted at the 6- or 8-position of the adenine ring have since been shown to serve as substrates for the phosphorylation of L-met-S-sulfoximine and thereby can be introduced specifically into active-sites of the enzyme as structural probes. The distance between active sites of the enzyme has been measured by fluorescence energy transfer taking advantage of the essentially irreversible binding of various ADP analogs at neutral pH when bound with L-met-S-sulfoximine phosphate and Mn^{2+} at active sites. For this purpose, we used two fluorescent donors with an average of 1 and 2 fluorescent ligands per dodecamer and two acceptors attached to either the 6- or 8-positions of ATP on the remaining active sites. Control samples of enzyme contained either of the two fluorescent donors with the remaining active sites covered with underivatized ATP. The distances calculated using the four combinations of donors and acceptors agreed well and indicated that the active sites on glutamine synthetase are 49 or 57 Å apart, if transfer occurs to a single acceptor or to two equidistant acceptors, respectively. A distance of 60 Å is close to the maximum distance possible between centers of active sites located on the outer edges of subunits within a hexagonal ring. Assuming isologous bonding between opposing subunits from the two hexagonal rings, the maximum distance between centers of these active sites is ~ 75 Å. Thus, there is little contribution from isologously bonded subunits in energy transfer; rather, transfer apparently occurs between heterologously bonded intra-ring subunits from active sites located very close to the outer edges of the hexagonal rings away from the central lateral plane of the dodecamer.

The mercaptonucleotides (6-S-ATP and 8-S-ATP) also can form very stable coordination complexes with aquoglycyl-L-methionyl platinum (II) and these have been used to introduce electron dense probes at active sites of glutamine synthetase for electron microscopic analyses and for X-ray crystallographic structural analysis. Also, the 6-S-AMP group of the enzyme adenylylated enzymatically with 6-S-ATP will react with the aquo-Pt(II) compound, resulting in electron dense markers at adenylylation sites. Scanning transmission electron microscopy (STEM) studies are in collaboration with J. Hainfeld, J. S. Wall, and J. J. Lipka at the Brookhaven National Laboratory. Preliminary results reported last year indicated that STEM image analysis could detect positions of increased mass in the 8-S-ADP·Pt(II) enzyme located near the outside edge of the hexagonal rings viewed along the 6-fold axis of symmetry. This result is in agreement with energy transfer measurements. However, the Brookhaven group has had difficulty in reproducing their initial result with freshly prepared samples and this has delayed publication. Further refinements in the analysis of 5-10 Å electron scatter data and new sample preparations are being tested now. The 8-S-ADP·Pt(II) derivative has been introduced also into all active sites of unadenylylated glutamine synthetase from Salmonella typhimurium and this derivatized enzyme has been returned to Dr. David Eisenberg at UCLA for the crystal growth necessary for X-ray crystallographic structural analysis.

Covalent labeling of ATP sites of E. coli glutamine synthetase with 5'-p-fluorosulfonylbenzoyladenosine (FSBA) enabled us to isolate a 4-carboxybenzenesulfonyl (CBS)-labeled peptide of ~ 4600 M_r after cyanogen bromide cleavage. This peptide contains no cysteine, tyrosine, serine, or tryptophan residues and does not contain the histidine residue oxidized by treatment of glutamine synthetase with mixed-function oxidase systems or the 5'-AMP group introduced by adenylylation, although these groups are near the active-site in the native, folded subunit structure. The FSBA-labeled peptide spans residues 9-48 from the N-terminal end of the subunit polypeptide chain and sequence analysis by automated Edman degradation in the laboratory of R. L. Heinrikson (University of Chicago) identified CBS-lysine at position 47. Thus, lysyl residue 47 appears to be near the subunit γ-phosphate binding site for ATP in the native glutamine synthetase subunit structure. Predictions of secondary structure based on amino acid sequence data suggest that Lys-47 is the C-terminal residue of an α-helix containing 20 residues, located just prior to a turn of the polypeptide chain.

Active-site ligand and metal ion interactions with mammalian octameric glutamine synthetase from bovine brain are being studied. This protein undergoes structural changes upon binding Mn^{2+} or Mg^{2+} or the activator, Cl^-. Also, two metal ion sites per subunit apparently must be saturated for expression of activity, which is the same as the Me^{2+} requirements of the E. coli enzyme. Possible physiological activators and inhibitors of the brain enzyme are being tested. In addition, quaternary structural restraints of this enzyme are being investigated using the inactivating ligands, Mn^{2+}, ATP, and L-met-S-sulfoximine. A specific covalent labeling of nucleotide substrate sites with various ATP analogs is being attempted also.

The release of Zn^{2+} from aspartate transcarbamoylase (ATCase; c_6r_6) upon challenge by p-hydroxymercuriphenylsulfonate (PMPS) has been studied using the sensitive, high-affinity metallochromic indicator 4-(2-pyridylazo)resorcinol at pH 7.0. When the -SH group of each catalytic (c) chain is protected, 1 Zn^{2+} is released for every 4 eq of PMPS added to ATCase during titration of the 24 -SH groups of regulatory (r) chains. Moreover, the release of Zn^{2+} is a linear

function of PMPS added, indicating that the rate-limiting step in Zn^{2+} release is mercurial attack on the first of the 4 r -SH groups bonded tetrahedrally to Zn^{2+} near c:r contacts. Dissociation of ATCase is linked to Zn^{2+} release and mercaptide bond formation; e.g., upon addition of 4 eq of PMPS in the absence of phosphate, 1/6th of ATCase is dissociated to c_3 and r_2 subunits at ~ 83% of the rate of Zn^{2+} release. Up to 4 eq of PMPS/ATCase, the release of Zn^{2+} is first order in [PMPS] and is virtually independent of [ATCase] with an activation energy (E_a) of 18 kcal/mol. At large excesses of PMPS, stopped-flow traces show a lag period followed by pseudo-first-order release of Zn^{2+} from ATCase and the reaction order in [PMPS] = ~ 1.3. Under these conditions, PMPS has a chaotropic effect on ATCase and E_a is lower. A participation of nonthiol protein groups of ATCase in mercurial binding is suggested by kinetic data. Furthermore, mercurial-promoted Zn^{2+} release is > 3000-fold faster from r_2 subunits than from ATCase. In conclusion, the disruption of 1 Zn^{2+} site in an ATCase molecule by organomercurial attack triggers the rapid reaction of all r chain -SH groups in the same molecule resulting in complete Zn^{2+} release and dissociation into c_3 and r_2 subunits. Thus, the intactness of Zn^{2+} binding clusters in an ATCase molecule is essential for thermodynamic stabilization of r and c chain interactions responsible for the allosteric properties of this enzyme. Current studies on the binding of Zn^{2+} to isolated regulatory subunits relate directly to the mechanism of ATCase assembly in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00201-13 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism of the Branched-Chain Amino Acids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Michael Poston Research Chemist LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A study of the metabolism of the branched-chain acids has revealed a pathway of metabolism of leucine that is catabolic in bacteria and appears to be synthetic in humans. The pathway depends upon the activity of the enzyme, leucine 2,3-aminomutase, an enzyme dependent upon adenosylcobalamin as a cofactor. Other enzymes which function in the pathway are 3-leucine transaminase, coenzyme A transferase, and thiolase. The relative carbon flux through this pathway and the pathway which is independent of cobalamin greatly favors the independent pathway in brain, heart, kidney, and liver. In the testis, however, the cobalamin-dependent pathway accounts for over forty percent of the carbon flux. This suggests that the metabolism of leucine may play an important role in this organ. The relationship between enzyme activity and various disease states such as pernicious anemia and inborn errors of metabolism will be examined.

Project Description:

Objectives: The catabolism of the branched-chain amino acids, leucine, isoleucine, and valine remains incompletely understood. Although much information that is available has been derived from the study of inborn errors of amino acid metabolism, studies of bacterial fermentation and of animal tissues have given additional information about the catabolism of these amino acids. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids, to examine the enzymes responsible for the various metabolic steps in these fermentations, to explore the distribution of these pathways in other species, to examine the enzymes in these other species, and to examine the implications of these pathways in human metabolism.

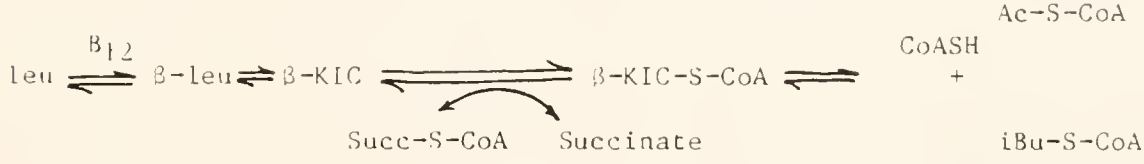
Major Findings: As previously reported, when cells or extracts of several clostridia, especially Clostridium sporogenes and Clostridium lentoputrescens, are incubated with L-leucine, several metabolic products are formed that are consistent with the metabolic pathway reported in mammals. However, the production of isobutyrate could not be explained by previously postulated pathways. Evidence was accumulated to support the pathway:

$$\alpha\text{-leucine} \rightleftharpoons \beta\text{-leucine} \rightleftharpoons \beta\text{-ketoisocaproate} \rightleftharpoons \text{acetate} + \text{isobutyrate}$$

The first step is catalyzed by the enzyme, leucine 2,3-aminomutase, which requires coenzyme B_{12} [adenosylcob(III)alamin]. This enzyme has been partially purified from a variety of sources (clostridia, spinach, potatoes, and sheep liver), but it has proven to be remarkably unstable after partial purification. It has been measured in human liver, hair roots, and leukocytes.

Subsequent work has shown that the pathway as originally conceived was incomplete. The project of the transamination, β-ketoisocaproate, is not cleaved directly, but must first be converted to the coenzyme A thioester. This is catalzyed by the enzyme, coenzyme A transferase, and uses succinyl-Co-A as the cosubstrate. The resulting thioester is then cleaved by a thiolase. Both the coenzyme A transferase and the thiolase have been purified and shown to be the well-characterized enzymes already described.

The pathway as it is now understood may be represented as shown below:



It has been a continuing question as to the relative importance of this pathway: what is the proportion of leucine that is metabolized in this pathway and how much is metabolized in the cobalamin-independent pathway? In order to examine this, organs from young adult rats were homogenized and exposed to L-[1-^{14}C]leucine. The proportion of the metabolism in the independent pathway was revealed by the amount of radioactive CO_2 evolved, whereas the volatile anionic fractions were a measure of the B_{12}-dependent path. It was found that liver and kidney utilized the independent pathway sending less than 1% through

the dependent one. The brain had 1.4% of the total flux of leucine carbon in the dependent pathway, the heart had nearly 5%. Testis, on the other hand, used the dependent pathway to a very significant degree, sending over 40% of the total flux in that direction.

Examination of the testis using various enzymic and buoyant density techniques suggests that the principal activity in the dependent pathway is located in the interstitial cellular component of the testis; the activity of marker enzymes and the B_{12}-dependent pathway indicates that the site of this metabolism is in the Leydig cells of the interstitial tissue. This suggests that the metabolism of leucine via the cobalamin-dependent pathway may have an important relationship to the endocrine tissue in this organ.

Efforts to measure and characterize the β-leucine transaminase have not been successful. A radioactive substrate was prepared in which the label was in carbon 2 of the skeleton of β-leucine. When this was used with sheep liver crude extract, there was a clear indication that the product was the same as that seen from radioactive α-leucine. Since only a small amount of the substrate could be prepared, a search for a more efficient synthesis of radio-labeled β-leucine or of β-ketoisocaproate has been initiated, with only limited success.

Significance to Biomedical Research and the Program of the Institute: This study impinges on several areas of medical concern: the mode of action of vitamin B_{12} in its metabolic roles, the metabolism of amino acids, and nutrition. The mode of action of B_{12} is imperfectly understood, but its importance in hematopoiesis and in the maintenance of proper neurological function is exemplified in the disease of its metabolic deficiency, pernicious anemia. Prior to these studies, only two B_{12}-dependent enzymes had been demonstrated in man; leucine 2,3-aminomutase is now the third. The finding that the endocrine tissue of rats has a strong metabolism in the B_{12}-dependent pathway may have significance in the understanding of some human metabolic problems. Several inborn errors have been involved with amino acid metabolism and the effects of these errors may be devastating to the well being of humans, especially in the instances of maple syrup urine disease, isovalericacidemia, and disorders of the catabolism of short-chain acids. The β-leucine pathway may be involved in some of the syndromes associated with these inborn errors.

Proposed Course: The enzymes of the β-leucine pathway will be further purified and characterized. Special effort will be given to the purification of the transaminase and the aminomutases. The relationship of the metabolism of leucine via the β-leucine pathway to the endocrine tissue of the testis will be examined.

Publications:

Poston, J. M.: The relative carbon flux through the α- and β-keto pathways of leucine metabolism. J. Biol. Chem. 259: 2059-2061, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00202-13 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Kinetics, Regulation and Mechanism of Biochemical Reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
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	Charles Y. Huang	Research Chemist	LB, NHLBI
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COOPERATING UNITS (if any)

E. Korn, Laboratory of Cell Biology, NHLBI; R. S. Adelstein, Laboratory of Molecular Cardiology, NHLBI; P. Greengard, Rockefeller University, New York; J. Vandenheede, Katholieke Universitiet, Belgium

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.3	2.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) The Mg(II)-ATP-dependent phosphoprotein phosphatase in skeletal muscle is inhibited by a cAMP-dependent protein kinase (type II). This inhibition is due to the binding of the dissociated regulatory subunit to the phosphatase. Although the catalytic subunit of the cAMP-dependent protein kinase is capable of phosphorylating the modulator subunit of the phosphatase, unlike the kinase F_a it fails to activate the inactive phosphatase.

(2) In an attempt to identify endogenous brain substrates for the Ca(II)-calmodulin-dependent protein phosphatase, the catalytic efficiencies (k_{cat}/K_m) for several well characterized phosphoproteins purified from brain were evaluated. The results indicated that DARPP-32, G-substrate and protein K.-F. are potential substrates for the phosphatase.

(3) The phosphorylation and dephosphorylation of smooth muscle myosin involves cooperative interaction between two heads when myosin is polymerized into filaments; but with monomeric myosin both phosphorylation and dephosphorylation occur randomly.

Project Description:

Objectives:

(1) Utilizing the physical-chemical approach to study the kinetics, mechanism, and regulation of biochemical activity. In particular, the biochemical action between the substrates, metabolites, and enzymatic activity of glutamine synthetase from Escherichia coli will be elucidated. In addition, fast kinetic techniques which are useful in these studies will be improved.

(2) Theoretical analysis of the cyclic cascade system with respect to its properties and function in the metabolic regulation of key enzymes will be continued.

(3) Isolation of the regulatory proteins to allow detailed mechanistic studies, and experimentally verify the validity of the cyclic cascade model described in (2).

(4) To search for the natural substrates of calmodulin activated phosphoprotein phosphatase.

Major Findings:

I. Analysis of the Cyclic Cascade System Involving Type II cAMP-dependent Protein Kinase and Mg(II)ATP-dependent Phosphoprotein Phosphatase (also see Stewart Jurgensen's Annual Report)

Theoretical analysis of cyclic cascade systems revealed that high sensitivity in response to changes of a given effector can be achieved if the effector involved can exert an opposite effect on the two opposing converter enzymes. The study of a major phosphatase in skeletal muscle, the Mg(II)ATP-dependent phosphoprotein phosphatase, revealed that cAMP-dependent protein kinase (type II) causes inhibition of the phosphatase. The inhibition was shown to derive from the binding of the dissociated regulatory subunit to the phosphatase. Although the catalytic subunit of the cAMP-dependent protein kinase is capable of phosphorylating the modulator subunit, unlike the kinase F_a it fails to activate the inactive phosphatase. The inhibition reaction can be reversed by the addition of excess catalytic subunit of the kinase which binds the regulatory subunit to form the holoenzyme. Addition of cAMP which causes the dissociation of the holoenzyme results in restoring the inhibition of the phosphatase.

This observation provides a unique role for the regulatory subunit of cAMP-dependent protein kinase. The observed inhibition of phosphatase together with the cAMP-dependent protein kinase provides a highly sensitive mechanism in response to changes in cAMP concentration for cyclic cascade systems which utilize both cAMP-dependent protein kinase and Mg(II)ATP-dependent protein phosphatase as converter enzymes.

II. Identify Endogenous Brain Substrate for Ca(II)-Calmodulin-dependent Protein Phosphatase (with C. Y. Huang)

Ca(II)-calmodulin-dependent protein phosphatase (also known as calcineurin) found in several tissues is highly concentrated in mammalian brain. In an attempt to identify endogenous brain substrates for this enzyme, kinetic analyses of the dephosphorylation of several well characterized phosphoprotein purified from brain were carried out. The proteins studied were: G-substrate, a substrate for cGMPdependent protein kinase; DARPP-32, a substrate for cAMP-dependent protein kinase; Protein K.-F., a substrate for a cyclic nucleotide- and Ca(II)-independent protein kinase; and synapsin I, a substrate for cAMP-dependent (site I) and a Ca(II)-calmodulin-dependent (site II) protein kinase. Calcineurin dephosphorylated each of these proteins in a Ca(II)-calmodulin-dependent manner. Similar K_m values were obtained for each substrate: G-substrate, 3.8 μM; DARPP-32, 1.6 μM; Protein K.-F., approximately 3 μM ($S_{0.5}$); synapsin I (site I), 7.0 μM; synapsin I (site II), 4.4 μM. However, significant differences were obtained for the maximal rates of dephosphorylation. The k_{cat} values were: G-substrate, 0.41 S^{-1},; DARPP-32, 0.2 S^{-1}; Protein K.-F., 0.7 S^{-1}; synapsin I (site I) 0.053 S^{-1}; synapsin I (site II), 0.04 S^{-1}.

Comparisons of the catalytic efficiency (k_{cat}/K_m) for each substrate indicated that DARPP-32, G-substrate and Protein K.-F. are all potential substrates for calcineurin *in vivo*.

III. Mechanistic Study of Phosphorylation and Dephosphorylation of Smooth Muscle Myosin (with R. S. Adelstein)

It is known that the 20,000 dalton light chain of both heads of a smooth muscle myosin or heavy meromyosin (HMM) molecule must be phosphorylated before the MgATPase activity of either head can be activated by actin. The two heads of HMM appear to be phosphorylated randomly at equal rates, while those of myosin are phosphorylated in a negatively cooperative manner. A study was carried out to investigate the cause of this difference between HMM and myosin. The results show that if myosin is first phosphorylated at high ionic strength (0.6 M KCl), where it is monomeric, and then assayed for MgATPase in 0.05 M KCl, the data support a model where the two heads are phosphorylated randomly with equal rates. The data for the dephosphorylation of fully phosphorylated myosin, both in a filamentous and monomeric state, are best explained by a model where dephosphorylation of one head is sufficient to deactivate the entire molecule. With monomeric myosin, the dephosphorylation occurs randomly with equal rates, whereas with filamentous myosin, the dephosphorylation of HMM and its MgATPase activity is more complex and is consistent with a postively cooperative dephosphorylation mechanism.

The phosphorylation of HMM and monomeric myosin proceeded via a first-order reaction. However, when phosphorylation is carried out in low ionic strength (0.02 M KCl), where myosin is present as filaments, the time course consists of two exponential functions in which the rate of one myosin head is 6-10 times faster than the other head which is located on the same molecule. This suggests that when myosin is polymerized into filaments, the two previously indistinguishable heads either become nonequivalent or are subject to head-head interaction leading to a negatively cooperative phosphorylation.

Significance to Biomedical Research and the Program of the Institute:

The overall objective is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties, and to elucidate principles of interaction between effectors, regulators, and proteins. This knowledge is instrumental in controlling the function of a specific enzyme by designing an effector or enzyme suicide substrate.

Proposed Course:

(1) To further expand the cyclic cascade model to substrate cascade and more complex enzyme cascade systems; and to further analyze the kinetic aspect of the cyclic cascade system.

(2) To study the mechanism of the cascade reactions which regulate the state of adenylylation for glutamine synthetase. We plan to study the protein-protein interaction between P_{II} protein and ATase, UTase, and UR, and ATase-glutamine synthetase interaction, by physical, chemical, and immunological methods, to test the validity of the bicyclic cascade model, and to study the role of the effectors in this cascade system.

(3) To further explore the physical, chemical, and immunological properties of unadenylylated and adenylylated glutamine synthetase. In particualr, we will utilize the fast reaction techniques, NMR, ESR, and fluorescence polarization methods to elucidate the roles of effectors and to identify the intermediates in the catalytic cycle. Laser light scattering will be used to study protein-protein interactions.

(4) To investigate the activation mechanism of cAMP-dependent protein kinase and reaction mechanism of phosphoprotein phosphatase.

Publications:

Jurgensen, S., Shacter, E., Huang, C. Y., Chock, P. B., Yang, S.-D., Vandenheede, J. R., and Merlevede, W.: On the mechanism of activation of the ATP·Mg(II)-dependent phosphoprotein phosphatase by kinase F_a. J. Biol. Chem. 259: 5864-5870, 1984.

Sellers, J. R., Chock, P. B., and Adelstein, R. S.: The apparently negatively cooperative phosphorylation of smooth muscle myosin at low ionic strength is related to its filamentous state. J. Biol. Chem. 258: 14181-14188, 1983.

Rhee, S. G., and Chock, P. B.: Purification and characterization of uridylylated and unuridylylated forms of regulatory protein P_{II} involved in the glutamine synthetase regulation in Escherichia coli. Isozymes Curr. Top. Biol. Med. Res. 8: 141-153, 1983.

Shacter-Noiman, E., Chock, P. B., and Stadtman, E. R.: Protein phosphorylation as a regulatory device. Phil. Trans. R. Soc. Lond. 302: 157-166, 1983.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: Nucleotidylation involved in the regulation of glutamine synthetase in E. coli. In Freedman, R. (Ed.): The Enzymology of Posttranslational Modification of Proteins, Vol. II, 1984, in press.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: E. coli glutamine synthetase. Meth. Enzymol. 1984, in press.

King, M. M., Huang, C. Y., Chock, P. B., Nairn, A. C., Hemmings, H.C., Jr., Chan, J. K. F., and Greengard, P.: Mammalian brain phosphoprotein as substrates for calcineurin. J. Biol. Chem. 259: 8080-8083, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00203-11 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Cellular Regulation of Enzyme Levels

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Cynthia N. Oliver	Staff Fellow	LB, NHLBI
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- xx ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are actively studying the regulation of intracellular enzyme level in relation to protein turnover and aging. We have previously characterized the degradation of Escherichia coli glutamine synthetase (GS) and have presented evidence for a two-step process involving inactivation followed by proteolysis (R. L. Levine, et al., 1981, Proc. Nat. Acad. Sci. U.S.A. 78, 2120-2124). Furthermore, we have characterized several enzymic mixed-function oxidation systems which catalyze inactivation of GS. The generality of the oxidative inactivation reaction is supported by the demonstration that several key metabolic enzymes other than GS are inactivated in a qualitatively similar manner by at least two enzymic mixed-function oxidation (MFO) systems, namely, microbial NADH-oxidase and the cytochrome P-450 system. We are currently examining the possible physiological role of MFO catalyzed enzyme inactivation (modification) in biological systems. We report here that activated neutrophils and HL-60 cells inactivate exogenous GS and GS in E. coli. Activated neutrophils also inactivate their endogenous enzymes and generate DNPH-reactive material. The oxidative modification of proteins by activated neutrophils may represent a possible mechanism of their bactericidal action as well as a mechanism by which these cells mediate host cell damage during chronic inflammation. Furthermore, several enzymes which we have found to be inactivated by the MFO systems have been found by others to accumulate in an inactive or less active forms during aging (L. Fucci et al., 1983, Proc. Nat. Acad. Sci. U.S.A. 80, 1521-1525.

We have also observed an increase of DNPH-reactive material in cultured fibroblasts from normal individuals over an age range of 60 years. In addition, DNPH-reactivity is markedly increased in fibroblasts from Werner's and progeria patients compared to age-sex matched controls. These are genetic diseases associated with accelerated aging. These results suggest that oxidatively modified proteins may accumulate in cells as a function of normal or abnormal aging.

Project Description:

Objectives: This project involves continuing studies on the oxidative inactivation of glutamine synthetase (GS) and other key metabolic enzymes by enzymic mixed-function oxidation (MFO) systems. The major objective of studies undertaken in the past year is to examine the possible physiological role of MFO-mediated enzyme inactivation (modification) in biological systems. Two model systems have been chosen for these studies. The first system involves the ability of activated neutrophils, neutrophil-like cells, and macrophage-like cells to inactivate bacterial enzymes as a possible mechanism of their bactericidal action. The second system involves an attempt to determine whether oxidative modification of enzymes is associated with either the normal or abnormal process of aging.

Major Findings:

Neutrophil Studies. The process of bacterial enzyme inactivation may be functionally important in host defense against bacterial infection in higher organisms. Mature polymorphonuclear leukocytes (neutrophils) for example are scavenger cells in higher organisms which are capable of ingesting and killing bacteria. These cells can be activated chemically (as well as by bacteria) by a variety of compounds including bacterial fmet-peptides such as fmet-leu-phe, latex beads, plant lectins, miscellaneous particles, complement and phorbol esters such as phorbol myristate acetate (PMA) to undergo a respiratory burst. This process is characterized by a shift from glycolysis to the pentose phosphate pathway, an increased uptake of oxygen, activation of a membrane associated NADPH-oxidase, and an increased production of activated oxygen species. During this respiratory burst (oxidative burst) neutrophils are capable of ingesting and killing large numbers of bacteria. Studies have shown that the activated oxygen species produced during the respiratory burst are diffusable and may be instrumental in bacterial target cell damage and death (Babior, B. M., 1978, N. Engl. J. Med. 298, 659-668; Weiss, S. J., 1980, J. Biol. Chem. 255, 9912-9917. Two enzymes are thought to play a major role in the generation of active oxygen species and bacterial killing, namely, a myeloperoxidase associated with neutrophil primary granules and a membrane associated NADPH-oxidase which is inactive in resting neutrophils and becomes activated during the respiratory burst. It is well known that patients with chronic granulomatous disease (CGD) ingest bacteria normally but exhibit a defective respiratory burst and are unable to kill bacterial efficiently. As a result, CGD patients often suffer from bouts of severe recurring bacterial infections. Activated neutrophils from these patients are deficient in NADPH-oxidase activity and these findings strongly implicate NADPH-oxidase (or an early activation step) in neutrophil mediated bacterial cell killing. Likewise, neutrophils from patients with hereditary myeloperoxidase deficiency kill some microorganisms much less efficiently than normal neutrophils. Because we have been able to show that another NADH-oxidase (microbial NADH-oxidase) is capable of inactivating a vareity of key metabolic enzymes *in vitro*, we have undertaken studies to determine whether activated neutrophils can inactivate bacterial enzymes as a possible mechanism of bacterial cell killing. For these studies, we have used neutrophil-like continuous cell-line (HL-60 cells), a macrophage-like continuous cell line (U-937 cells), and freshly isolated human neutrophils.

Experiments with HL-60 Cells. Initial studies with neutrophil-like cells were carried out with continuous HL-60 cells. These cells were derived form a

patient with promyelocytic leukemia and are passaged as a promyelocyte stem cell which can be differentiated into neutrophil-like cells by a vareity of exogenous agents such as dimethylsulfoxide (DMSO) or retinoic acid (RA). During differentiation, these cells become respiratory burst competent and can be stimulated to undergo a respiratory burst which is qualitatively similar to normal freshly isolated neutrophils. Early studies (Annual Report, 1983) with these cells indicated that culture conditions, particularly serum composition was extremely important in obtaining reproducible differentiation and respiratory burst activity. After screening several commercial sources of fetal bovine serum (FBS), we located a batch of highly defined serum which contains low endotoxin levels (0.06-0.1 ng/ml), relatively high insulin levels (10 μunits/ml) and high transferrin levels (198 mg/dl). Cells grown on a medium containing this serum (10% by volume and 1% DMSO) are approximately 70% differentiated (as judged by formazan deposition following nitroblue tetrazolium reduction) and 90% viable (as judged by trypan blue exclusion) in four days. HL-60 cells differentiated in this way exhibit about 25-50% of respiratory burst activity (as judged by cytochrome C reduction following stimulation with 10^{-6} M phorbol myristate acetate, PMA) of freshly isolated normal neutrophils.

Differentiated (DMSO-treated), stimulated (PMA-treated) HL-60 cells inactivate purified exogenous E. coli GS as well as GS in intact E. coli cells. HL-60 cells differentiated with 10^{-6} M RA inactivate GS in the presence of PMA, but not in the presence of fMLP (f-met-leu-phe) or fNLP (f-norleu-leu-phe). These results are consistent with the observation that RA-differentiated cells lack the receptor for the chemotactic peptides. HL-60 cells differentiated with 1% DMSO inactivate GS in the presence of PMA, fMLP, or fNLP. (fNLP was used in these studies because methionine sulfoxide formation in fMLP during respiratory burst inactivates the chemotactic peptide.)

Treatment of differentiated (DMSO-treated) HL-60 cells with chemotactic peptides (fMLP or fNLP) leads to the generation of small amounts of H_2O_2 but no detectable O_2^-, whereas treatment of the same cells with PMA leads to the production of both O_2^- and H_2O_2. Undifferentiated HL-60 cells or differentiated unstimulated HL-60 cells do not generate O_2^- and H_2O_2 and do not inactivate E. coli GS. PMA at concentrations 10^{-4} M, 10^{-5} M, and 10^{-6} M has no effect on GS activity. Catalase, Mn^{++}, EDTA, and o-phenanthroline only partially inhibit GS inactivation catalyzed by stimulated HL-60 cells. These results are clearly different from the in vitro enzymic MFO systems in which GS inactivation can be completely blocked by these agents.

Experiments with U-937 Cells. Another continuous cell line, a macrophage-like cell line, U-937 cells, was also used in these studies. These cells were isolated from a patient with histocytic lymphoma. They are easy to propagate and need not be differentiated to become respiratory burst competent. Nevertheless, the production of both O_2^- and H_2O_2 is low, but is enhanced by treatment with 10^{-3} M dibutyryl cyclic AMP, dbcAMP for 2 days. When these cells are stimulated with PMA, they also inactivate exogenous purified E. coli GS and the time course of inactivation parallels that of respiratory burst. Characteristically macrophages and macrophage-like cells undergo a respiratory burst of longer duration and much lower intensity compared to neutrophils.

Experiments with Neutrophils. Although large quantities of HL-60 cells and U-937 cells could be readily obtained, the relatively low respiratory burst activity and GS inactivation activity was often difficult to quantitate. In addition, one might question whether these cells exhibited activity characteristic of normal neutrophils and macrophages. For these reasons, similar studies were carried out with freshly isolated neutrophils obtained either from leukophoresis leukopacks or from freshly drawn heparinized blood. The cells were isolated by Ficoll-Paque discontinuous density gradients followed by dextran sedimentation. Neutrophils from leukophoresis leukopacks were usually partially activated as a result of the leukophoresis products. When stimulated with 10^{-9} M PMA, these cells generated approximately 30 nmols of H_2O_2 and 1330 nmols of O_2^-, whereas neutrophils isolated from freshly drawn blood generated 68 nmols H_2O_2 and 2448 nmols of O_2^- under the same conditions. Viability in the leukopack neutrophils was not reduced. Treatment of neutrophils from freshly drawn blood with fMLP led to the generation of only 12 nmols of H_2O_2 and no detectable O_2^-. Neutrophils treated with either PMA or fMLP catalyzed the inactivation of exogenous purified GS and GS in E. coli. When PMA was used, GS inactivation was partially inhibited by catalase, SOD, Mn^{++}, EDTA, and o-phenanthroline. At pH 6.0, Mn^{++} actually stimulated inactivation, but at pH 7.4, Mn^{++} partially inhibited inactivation. The reason for this result is not clear. SOD had no effect on neutrophil mediated GS inactivation in the presence of fMLP. This observation is consistent with the apparent lack of O_2^- production in the presence of fMLP. The cytochrome C reduction assay, however, depends on the extracellular production O_2^- by neutrophils. If neutrophils are broken by sonication in the first 5 minutes of fMLP treatment, reduction of cytochrome C is observed. Although these results are difficult to interpret, the possibility remains that stimulation with PMA may lead to release of O_2^- generating enzymes to the plasma membrane (extracellular compartment), whereas stimulation with fMLP may lead to release of O_2^- generating enzymes to phagolysosomes (intracellular compartment). These results suggest, nevertheless, that at least two pathways exist for neutrophil activation both of which may lead to enzyme inactivation (oxidative modification).

There have been numerous reports in the literature that activated neutrophils damage fibroblasts, endothelial cells, erythrocytes, and other neutrophils (summarized by Test, S. T., Weiss, S. J., 1984, J. Biol. Chem. 259, 399-405). Suicidal damage of neutrophils is in fact suggested by the observation that PMA stimulated neutrophils accumulate oxidatively modified protein as judged by reactivity with the carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH). Under the same conditions, the levels of four enzymes, namely, LDH, PGK, G-3-PDH and PK are decreased (50-75%) compared to control cells.

Proposed Course:

The results obtained with both freshly isolated neutrophils and HL-60 cells have suggested the existence of at least two pathways of neutrophil activation. If these pathways are closely related, definitive dissociation by manipulation of experimental conditions may be exceedingly difficult. We have demonstrated that stimulation of neutrophils with PMA or fMLP leads to inactivation of GS and because we are interested in the modification(s) which lead to inactivation, experiments have been designed to determine whether neutrophils stimulated with these agents generate modifications which are similar to those observed in MFO systems in vitro. These studies will be carried out with synthetic amino acid

polymers as well as with selected enzymes. Additional studies are planned to determine whether the modifications of enzymes generated by activated neutrophils are more susceptible to proteolysis by proteases which have been isolated because they exhibit selectively for MFO modified proteins (in vitro) compared to native proteins. As a corollary to these studies, we plan to investigate the possibility that neutrophil activation leads to the activation of a protease (or proteases) which exhibit selectivity for oxidatively modified proteins.

It is tempting to speculate that bactericidal action of activated neutrophils may be due in part to the oxidative inctivation of key metabolic enzymes in bacteria, a process which may be independent of phagocytosis. In this context, experiments are planned to determine whether neutrophil-mediated enzyme inactivation in bacteria is associated with killing of bacterial cells. Moreover, because neutrophil activation has been implicated in host cell damage (endothelial cells erythrocytes, fibroblasts and other neutrophils), enzyme inactivation (oxidative modification) will be further investigated in these systems as a possible mechanism of neutrophil or macrophage-mediated cell damage during chronic inflammation.

Major Findings:

Aging Studies. Over the past several years, we have studied the mixed-function oxidation (MFO)-mediated inactivation of GS and other key metabolic enzymes. Levine has demonstrated (J. Biol. Chem., 258, 11823-11827, 1983) that GS inactivation is a highly specific process involving the oxidative modification of a histidine residue in each GS subunit and the generation of a carbonyl derivative of the protein which forms a stable hydrazone when treated with 2,4-dinitrophenylhydrazine (DNPH). Many of the enzymes which we have shown to be inactivated in vitro by MFO systems have been shown by others to accumulate in inactive or less active forms during cell aging. Because Werner's syndrome and progeria are genetic diseases associated with accelerated aging, studies have been undertaken to determine whether oxidatively modified proteins accumulate in fibroblasts of these patients compared to cells from normal individuals. Another disease, Alzheimer's Disease, is characterized by selective loss of functional enzyme activity and studies have been undertaken to determine whether this loss of enzyme activity is associated with the accumulation of oxidatively modified protein. Because protein carbonyl derivatives are generated by MFO-mediated inactivation reactions in vitro, DNPH-reactivity is used as a presumptive screening assay for oxidatively modified protein.

Experiments with Werner's Syndrome and Progeria Fibroblasts. Fibroblasts from 2 patients with progeria and 2 patients with Werner's syndrome as well as 12 fibroblast cultures from apparently normal individuals ranging in age from 13-84 years were obtained (Institute for Medical Research, Aging Cell Repository, Camden, New Jersey). Most of the normal cultures grew well giving rise to even cell sheets with smooth processes aligned in parallel arrays. Growth of Werner's and progeria fibroblasts was much more variable and typically exhibited large gaps in the cell sheet, tangled processes and clustered cell bodies with large vacuoles and granules. Cell cultures were grown to confluency in 85% Dulbecco's modified Eagle's medium and 15% nonheat inactivated fetal bovine serum. The cultures were washed several times in serum-free medium before harvest, harvested by scraping, washed and resuspended in phosphate buffered saline, pH 7.2.

Extracts were prepared either by light sonication or by manual homogenization. Extracts were assayed for protein and then treated with 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl. Following a series of extractions with organic solvents, the difference spectrum of the hydrozone mixture compared to samples treated with 2 N HCl alone was obtained. All cultures were compared at the same relative passage number and only small differences were observed in early passage cultures compared to late passage cultures. Some cultures have been passaged for over 20 population doublings.

Results from preliminary studies indicate that there is a difference of DNPH reactivity of proteins in cultures from normal individuals covering an age range of 60 years. More DNPH reactive material is present in fibroblasts from an 80-year old individual compared to a 13-year old individual. Progeria and Werner's fibroblast cultures possess markedly increased (2-fold) DNPH-reactive material compared to age and sex matched normal controls.

Experiments with Alzheimer's Disease. Two enzymes, choline acetyltransferase (CAT) and acetylcholinesterase (AChE) at least from electric eel, have a histidine associated with the catalytic site and both are known to be inactivated by copper in the absence of EDTA. These properties suggested that these enzymes might be likely candidates for MFO-mediated inactivation. In fact, when incubated with microbial NADH-oxidase, both CAT and AChE are inactivated. Moreover, Shinar et al. (1983, J. Biol. Chem. 258, 14778-14783) have demonstrated inactivation of AChE in the presence of ascorbate and copper. Alzheimer's Disease (Alzheimer's Disease/Senile Dementia Alzheimer's Type, AD/SDAT) is a neurological disorder (dimentia) associated with selective memory loss and progressive loss of cognitive function in otherwise healthy individuals. Recent evidence indicates that AChE and CAT are deficient in cortical presynaptic axons of patients with AD/SDAT and absent in cell bodies of nucleus basalis of Meynert (basal forebrain) which normally send cholinergic projections to frontal, medial, and paretal cortex. The neuropathology is characterized by neurofibrillary tangles and by the development of neuritic plaques composed of an "amyloid-like" material. The reduction of CAT and AChE in the presynaptic axons correlates with the severity of dementia and with the frequency of neuritic plaques. Moreover, during evolution of neuritic plaques, the amount of amyloid increases and the activity of AChE decreases. End-stage plaques lack AChE.

We have undertaken a collaborative study with Drs. D. L. Price and P. J. Whitehouse (Neuropathology Department, Johns Hopkins Medical School) to investigate the possibility that CAT and AChE might be oxidatively inactivated and may accumulate in neuritic plaques as denatured DNPH-reactive proteins. We have obtained approximately 40 coded samples and pilot experiments have been carried out with rat brains in order to define sufficiently sensitive assay conditions and extraction procedures. Preliminary studies have suggested that part of AChE activity is soluble and part of it is particulate and further preliminary studies are underway to determine whether the form or specific activity of these enzymes varies with compartmentation of the enzymes.

Proposed Course:

We are in the process of obtaining more normal fibroblast cultures as well as additional cultures from progeria and Werner's syndrome patients. In additio

to experiments designed to quantitate DNPH-reactivity, the activity of selected enzymes would be determined as a function of age of the donor and age of the culture. In other studies (B. Ahn, Annual Report 1984), we expect to purify several enzymes from erythrocytes in order to prepare high affinity (polyclonal) antibody. Using these antibody preparations, it may be possible to isolate enzyme mixtures (containing native and modified enzymes) and to determine whether enzyme modifications generated in vivo are similar to modifications catalyzed by MFO in vitro.

The Alzheimer's study involves 3 parts. The first part is to determine whether DNPH reactivity correlates with the loss of AChE and CAT and an increase in "amyloid-like" material in neuritic plaques of Alzheimer's patients compared to normal individuals. The second part would involve the use of specific antibody to AChE and CAT (antibody preparations to both enzymes are available) to isolate these enzymes and determine whether either or both enzymes are oxidatively modified. Finally, if it is possible to demonstrate oxidative modification of CAT and AChE in Alzheimer's brains, it might be possible to lesion the nucleus basalis of Meynert with a nonenzymic MFO system in experimental animals in order to study the devleopment of neuritic plaques and neuronal degeneration in the cortex. This might constitute at least a pseudo-animal model in which agents blocking or retarding this process could be tested.

Publications:

Oliver, C. N., Ahn, B., Wittenberger, M. E., and Stadtman, E. R.: Oxidative inactivation of enzymes: Implication in protein turnover and aging. In Ebashi, S. (Ed.): Cellular Regulation and Malignant Growth. New York, Academic Press, 1984, in press.

Rivett, A. J., Roseman, J. E., Oliver, C. N., Levine, R. L., and Stadtman, E. R.: Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. In Khairallah, E. A., Bond, J. S., and Bird, J. W. C. (Eds.): Intracellular Protein Catabolism. New York, Alan R. Liss, 1984, in press.

Oliver, C. N., Fulks, R., Levine, R. L., Fucci, L., Rivett, A. J., Roseman, J. E. and Stadtman, E. R.: Oxidative inactivation of key metabolic enzymes during aging. In Roy, A. K. and Chatterjee, B. (Eds.): Molecular Basis of Aging. New York, Academic Press, 1984, pp. 237-264.

Oliver, C. N.: Inactivation of enzymes by activated human neutrophils. Curr. Top. Cell. Regul., 1984, in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00204-17 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein Structure: Enzyme Action and Control

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator on the same Laboratory and Section.)

PI:	Ann Ginsburg	Chief, Section on Protein Chemistry	LB, NHLBI
Others:	Harold B. Pinkofsky	Staff Fellow (8/22/82-)	LB, NHLBI
	Sue H. Neece	Chemist (part-time 3/16/82-)	LB, NHLBI
	Marlana B. Blackburn	Staff Fellow (1/22/84-)	LB, NHLBI
	Philip G. Kasprzyk	Staff Fellow (6/24/84-9/14/84)	LB, NHLBI
	Steven Pease	Summer Student (5/29/84-6/22/84)	LB, NHLBI

Cooperating Units (if any)

M.R. Maurizi, NCI, Lab. Molecular Biology; J. Hainfeld, J.S. Wall, and J.J. Lipka, Biological Dept., Brookhaven National Lab., N.Y.; J.B. Hunt, Catholic University of America; R.L. Heinrikson, University of Chicago; H.K. Schachman, University of California, Berkeley; D. Eisenberg, University of California, Los Angeles

LAB BRANCH

Laboratory of Biochemistry

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.0	3.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) Nucleotide analogs have been synthesized for use as structural probes of glutamine synthetase from Escherichia coli in order to obtain intra- and inter-subunit distances in the dodecameric enzyme. Various analogs of ATP that are substituted at the 6- or 8-position of the adenine ring have been further modified with spectrophotometric and fluorometric probes or an electron dense Pt(II) marker for electron microscopy and for X-ray crystallographic studies. Active sites of this enzyme appear to be at the outer center edge of each subunit in the hexagonal rings; heterologously bonded intra-ring subunits have active sites ~ 57 Å apart (which is almost the maximum distance possible) and isologously bonded subunits from opposing rings have sites located at the exterior ~ 75 Å apart.

(2) Glutamine synthtase active sites also have been covalently labeled and lysyl residue 47 has been found to be near the subunit ATP binding site of the E. coli enzyme.

(3) Active-site ligand and metal ion interactions with mammalian glutamine synthetase are being studied to define structural and catalytic roles of divalent cations.

(4) Thermodynamic parameters for the sequential binding of active-site ligands to E. coli glutamine synthetase have been obtained by calorimetry, equilibrium binding, and fluorescene titrations.

(5) Studies on the mercurial-promoted release of Zn^{2+} from E. coli aspartate transcarbamoylase (ATCase) have been completed. Studies on the rebinding of Zn^{2+} ions to isolated regulatory subunits of this enzyme are in progress. The results relate to changes in catalytic and regulatory chain interactions in ATCase molecules responsible for the allosteric properties of this enzyme and to mechanisms of dissociation and assembly of this enzyme.

Project Description:

Objectives: (1) In general, to study conformation and stabilization changes of biologically important protein macromolecules promoted by the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. To study protein-metal ion interactions by kinetic and equilibrium methods to determine structural and catalytic roles of specific divalent cations. Spectrophotometric, fluorometric, ultracentrifugal, calorimetric, electrophoretic, chromatographic, enzyme kinetics, stopped-flow kinetics, and collaborative electron microscopic techniques are applied as required.

(2) To study the physical and chemical properties of unadenylylated and adenylylated glutamine synthetases (GS) from Escherichia coli, particularly with respect to correlating the regulation, structure and catalytic function of GS. The unusual properties of the complex formed when ADP, L-methionine-S-sulfoximine phosphate, and two divalent cations are bound to active sites of E. coli GS are being used to investigate the effects of these tightly bound active-site ligands on the tertiary and quaternary structure of the dodecameric enzyme and to obtain estimates of inter-subunit and intra-subunit spatial distances by introducing nucleotide analogs as structural probes at active sites and at adenylylation sites of the enzyme. Covalent modification of GS with other nucleotide analogs will locate this interaction site in the subunit primary structure.

(3) To study active-site ligand interactions with mammalian GS in order to detect any structural homology with E. coli GS. For this purpose, GS from bovine and ovine brain are being purified and characterized.

(4) To study Zn^{2+} bonding domains of aspartate transcarbamoylase (ATCase) from E. coli and of isolated regulatory subunits of ATCase in order to better understand the structural role of Zn^{2+} in the intact enzyme and in the assembly of ATCase from regulatory and catalytic subunits.

Major Findings:

(1) The use of nucleotide analogs as structural probes of E. coli GS: Spectrophotometric fluorometric, and electron microscopic studies. (Investigators: M. R. Maurizi and A. Ginsburg).

E. coli GS is composed of 12 identical subunits (M_r 50,000) arranged in 2 superimposed hexagonal rings. Each subunit has an active-site which binds substrates and 2 divalent metal ions and a regulatory site near the active-site which contains a tryosyl residue that can be covalently modified with AMP.

As summarized in last year's annual report, M. R. Maurizi has prepared various derivatives of ATP substituted at the 8- or 6-position of the adenine ring. These were shown to bind fairly tightly to GS ($K_A' > 10^5\ M^{-1}$) and to substitute for ATP in the auto-inactivation reaction of Mn·GS with L-met-S-sulfoximine (MSOX) at pH ~ 7 (J. Biol. Chem. 257, 4271-4278, 1982). With MSOX phosphate, 2 Mn^{2+}, and the corresponding analog of ADP tightly bound at a subunit active site ($K_A' > 10^{12}\ M^{-1}$) both intra- and inter-subunit contacts are strengthened (J. Biol. Chem. 257, 4271, 7246, 1982). By forming the inactive complex with various derivatives of ATP substituted in the 6- or 8-position,

specific probes have been introduced into the active sites of GS for spectrophotometric, fluorometric, electron microscopic, and X-ray crystallographic studies. In addition, some of these ATP analogs can be introduced into adenylylation sites by adenylyltransferase-catalyzed adenylylation to act as an additional structural probe of GS. Thus, specific well defined sites of GS can be labeled with structural probes without interference from nonspecific labeling.

The distance between active sites of dodecameric GS was measured by fluorescence energy transfer taking advantage of the essentially irreversible binding of ATP analogs in the presence of MSOX and Mn^{2+}. Two fluorescent ATP derivatives were used: the first was obtained by alkylation of 8-mercapto ATP with N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (IAEDANS) to produce AEDANS-ATP; the second was 1-N^6-etheno-2-aza ATP (aza-ε-ATP). The acceptors were obtained by alkylation of 6-mercapto or 8-mercapto ATP with 4-dimethylaminophanyl azophenyl-4-iodoacetamide to produce 6-Y-ATP or 8-Y-ATP, respectively. The procedure for the energy transfer experiments was first to obtain GS with an average of 1 and 2 fluorescent ligands per dodecamer by partially inactivating the enzyme with MSOX, Mn^{2+}, and limiting amounts of either AEDANS-ATP or aza-ε-ATP. The remaining active sites were then inactivated with MSOX, Mn^{2+}, and either 6-Y-ATP or 8-Y-ATP as acceptor or with underivatized ATP instead of acceptor for controls. Inactive protein derivatives were then gel filtered through P-10 columns to remove free and loosely bound ligands. The difference in fluorescence yield between the fully inactive complexes with ADP or with Y-ADP occupying most of the active sites was taken as a measure of fluorescence energy transfer. The efficiency of energy transfer was then used to calculate the distance between pairs of active sites according to the Förster equation, $R = R_o(E^{-1}-1)^{1/6}$. Results of experiments performed with an average of 1 and 2 fluorescent donors per dodecamer were averaged and the results are summarized in Table I.

Table I. Distances between Active Sites of Glutamine Synthetase

Donor/Acceptor	R_o[a] (Å)	Transfer Efficiency %	R[b] (1 acceptor site) (Å)	R[b] (2 acceptor sites) (Å)
AEDANS-ATP/6-Y-ATP	41.5	26	50	57
AEDANS-ATP/8-Y-ATP	41.9	22	52	59
Aza-ε-ATP/6-Y-ATP	42.7	32	49	56
Aza-ε-ATP/8-Y-ATP	43.2	37	47	55

[a] R_o is the critical transfer distance where the efficiency of energy transfer is 50%.

[b] R is the calculated distance between active sites of GS assuming that transfer occurs to either a single acceptor or to 2 equidistant acceptors on adjacent subunits.

The distances calculated using the four combinations of donor and acceptor agreed well (Table I) and indicated that the active sites on GS are in the order of 50-60 Å apart. Several assumptions have been made in interpreting the results of these experiments and in calculating the distances. For example, it was necessary to assign a value of 2/3 to the orientation factor, which is valid only for a random orientation between the emission dipole of the donor and the absorp-

tion dipole of the acceptor. As discussed by Stryer (Annu. Rev. Biochem. 47, 819, 1978), the probability of error in using this value can be reduced by obtaining average distance measurements with several different donors and acceptors. By using two fluorescence donors that are chemically and structurally different and two acceptors that are chemically the same but differ in their point of attachment to the nucleotide (and presumably orientation), we should have minimized the error in using the value of 2/3. It has also been assumed that energy transfer occurs between particular pairs of active sites and not between one active site and several others. As a result of this assumption, any calculated distance based on energy transfer is a minimum distance between sites, since transfer to several sites would lead to decreased fluorescence yield and would make it appear that energy transfer was more efficient. The effect of transfer from a single donor to two equidistant acceptors depends on the distance between donors and acceptors. If the apparent efficiency of transfer is less than 50%, the actual distance can be 10-25% higher than the calculated distance, whereas if the apparent efficiency is much greater than 50%, the actual distance could approach twice the calculated distance. In our measurements, the apparent efficiency of transfer was about 24% for AEDANS-ATP and about 34% for aza-ε-ATP. If transfer occurs to two adjacent acceptors, these efficiencies should be 12% and 17%, respectively, and the calculated distances between active sites are then 58 Å and 56 Å, respectively.

Negatively stained GS in electron microscopic images shows that the maximum distance across the hexagonal ring is ~ 140 Å and the distance between centers of adjacent subunits is ~ 45 Å. The distance between centers of superimposed subunits from opposite rings is also 45 Å. If one assumes that the subunits within a hexagonal ring are heterologously bonded, the distance between identical points at the exterior of the ring is 70 Å. However, the active site of a GS subunit must accommodate several substrates, two divalent metal ions, and a nearby adenylylation site. Measurements from several laboratories indicate that the minimum linear dimension of the active site can be approximated by a spherical volume of ~ 12 Å in diameter. Thus, the maximum distance between centers of active sites at the outer edge on adjacent subunits within a ring is close to 60 Å. Assuming isologous bonding between opposing subunits from the two rings, the maximum distance between centers of active sites located at the exterior is ~ 75 Å.

The calculated values for the distance between active sites are close to the maximum distance possible. If the maximum distance between active sites is 60 Å, the minimum efficiency of energy transfer (assuming no orientation problems) is 10% for AEDANS-ATP and 12% for aza-ε-ATP. Since there are two sites adjacent to each fluorescent ligand, the minimum transfer efficiencies are 20% and 24%, respectively (compared to the observed values of 24% and 34%). Thus, it appears that the active sites of GS subunits are located at or near the perimenter of the hexagonal rings. Since there is little contribution from yet the third site (i.e., the isologously bonded subunit), the active sites appear to be toward the outer edge of the dodecamer away from the central lateral plane of the dodecamer.

The results from energy transfer measurements are consistent with preliminary STEM (scanning transmission electron microscopy) results obtained in collaboration with J. Hainfeld, J. S. Wall, and J. J. Lipka (Biology Department at

Brookhaven National Laboratory) which show additional mass at the perimeter of GS when platinum (II) is specifically bound at active sites. This was accomplished by preparing a coordination complex between 8-S-ATP and aquoglycyl-L-methionyl platinum (II) and subsequently binding the Pt(II)-labeled ATP irreversibly in the presence of MSOX and Mn^{2+}. STEM analysis of parallel samples of inactive GS labeled with 8-S-ADP•Pt(II) complex and with 8-S ADP alone (or with ADP alone) were performed. The unstained images were stored as digitized electron scatter data in 10 Å square picture elements. Each image was centered and rotationally aligned by reference to a high resolution image of a stained GS particle. The images were summed and 6-fold rotationally averaged to produce the final average image for Pt-labeled and unlabeled samples. The difference between these average images corresponds to the amount and position of the increased mass due to the Pt(II). Initial analysis indicated 6 regions of increased mass located near the outside edge of the hexagonal rings viewed along the 6-fold axis of symmetry. These results strongly indicate that the regions of higher mass density can be detected by analysis of differences in electron scatter and that with further refinements this technique will allow an unprecedented localization of specific binding sites in GS.

However, a precautionary note must be added because the Brookhaven group has been unable to reproduce the initial result with subsequent 8-S-ADP•Pt(II) derivatives of GS which we have prepared, although Dr. Marlana Blackburn found by analysis that the newer samples also contained 1 eq of Pt(II) per subunit. We have supplied the Brookhaven group also with GS inactivated with a mercurial derivative of 8-S-ADP. Technical difficulties with the image analysis by STEM hopefully will be worked out in the near future.

We also have sent the 8-S-ADP•Pt(II) derivative of fully inactivated GS to Dr. David Eisenberg (UCLA) for X-ray crystallographic studies. In addition, we have just put 1 eq of 8-S-ADP•Pt(II) per subunit on the fully unadenylylated GS from an ATase-minus mutant of S. typhimurium for Eisenberg's group. Hopefully crystals of this derivative will grow to a sufficient size for X-ray crystallographic structural analysis. (Drs. Sue H. Neece and Marlana Blackburn collaborated in the latter studies).

(2) Covalent labeling of ATP sites of glutamine synthetase. (Investigators: H. B. Pinkofsky in collaboration with R. L. Heinrikson at the University of Chicago).

The ATP analog 5'-p-fluorosulfonylbenzoyladenosine (FSBA) inactivates dodecameric GS from E. coli with concomitant labeling of one site/subunit (Foster, W. B., Griffith, M. T., and Kingdon, H. S.: J. Biol. Chem. 246, 882-886, 1981). Cyanogen bromide cleavage of the FSBA inactivated enzyme in 70% HCOOH produced a large peptide (M_r = 4600) in ~ 75% yield containing N'-4-carboxybenzenesulfonyl-(CBS)lysine. The CBS-peptide was purified by high performance liquid chromatography on the basis of its relatively high UV absorbance at 246 nm ($\Delta\varepsilon \approx 19{,}000$ M^{-1} cm^{-1}). The appearance of the labeled peptide coincided with the loss of a peptide (lacking tyrosine and tryptophan) from the unmodified, active enzyme; the CBS-peptide also could be distinguished from cysteine-containing peptides labeled with thionitrobenzoate and from the adenylylated peptide. The identical peptide was labeled when the unadenylylated enzyme was inactivated with FSBA in the absence or presence of Mn^{2+} or Mg^{2+} (± L-glutamate) or when the enzyme was

adenylylated. Conformational differences among these enzyme forms therefore are not detected by affinity labeling of ATP sites. The FSBA-labeled peptide spans residues 9-48 from the N-terminal end of the subunit polypeptide chain, and sequence analysis by automated Edman degradation revealed that CBS-lysine was at position 47. Thus, lysyl residue 47 appears to be near the subunit γ-phosphate binding site for ATP in the native GS structure. The primary structure of the CBS-labeled peptide is:

```
Leu-Asn-Glu-His-Glu-Val-Lys-Phe-Val-Asp-Leu-Arg-Phe-Thr-Asp-Thr
 9                        15

Lys-Gly-Lys-Glu-Gln-His-Val-Thr-Ile-Pro-Ala-His-Gln-Val-Asn
25                    30

Ala-Glu-Phe-Phe-Glu-Glu-Gly-Lys-Homoser
40                    45        |
                                CBS
```

Secondary structure predictions for this sequence plus the following 10 residues places lysyl 47 at the C-terminus of a long, helical segment composed of 20 amino acid residues just prior to a turn in the subunit polypeptide chain. After forwarding this information to Dr. David Eisenberg at UCLA, such a helical segment at the outer edge of the subunit structure was tentatively identified in the X-ray crystallographic structural analysis of GS from S. typhimurium. Furthermore, the isologously bonded subunit in the opposing ring seemed to have a similarly located α-helical segment.

(3) Studies on the binding of active-site ligands and metal ions to GS from bovine brain. (Investigators: H. B. Pinkofsky in collaboration with M. R. Maurizi).

The native, catalytically active form of mammalian GS is an octomer. Despite extensive studies on this enzyme, the roles of Me^{2+} ions and activators in catalysis is poorly understood.

The purified bovine brain GS (400 U/mg in the Mn^{2+}-supported glutamyl transfer assay at pH 6.8 and at least 95% pure in SDS-slab gel electrophoresis) has a specific absorption coefficient at 280 nm of 1.50 ± 0.02 which alters published stoichiometries of Mn^{2+} binding. We also have found that the enzyme is stable for several months at 4°C when stored in the presence of 50 mM K-PO_4 (pH 6.8) and 1 mM EDTA in the absence of Cl^- and of Me^{2+} ions, which makes many studies now possible.

To explore the catalytic requirements, K'_m data for Mn^{2+}, Mg^{2+}, and ADP were obtained at pH 7.2 and saturation of 2 Me^{2+} sites is apparently necessary for catalytic activity expression as has been found for E. coli GS. Furthermore, a Job analysis of ADP-supported γ-glutamyl transfer activity indicates that all subunits of bovine brain GS express activity at 1 eq of ADP bound/subunit. Also, inactivation of the enzyme with Mn^{2+} or Mg^{2+} with ATP and MSOX resulted in the tight binding of 2 Me^{2+}, 1 ADP, and 1 MSOX phosphate per subunit. Mn^{2+} (K'_D = 45 μM) or Mg^{2+} (1-10 mM) quenches tryptophanyl residue fluorescence; Cl^- produces an additional quench (K'_D ~ 100 μM) which may relate to the activation

of the enzyme by Cl^-. This will be explored as well as a role of other potential activating anions. UV spectral perturbations of the brain GS produced by Mn^{2+} and Mg^{2+} binding have been measured. Peak-troughs at 292-299, 292-288, and 280-288 nm were observed in both Mn^{2+} and Mg^{2+}-induced difference spectra, but the magnitudes of the perturbations were less with Mg^{2+}. A value of $K'_A = 5 \times 10^3\ M^{-1}$ for Mg^{2+} binding was calculated from these data; however, the binding of Mn^{2+} is too tight to measure by this procedure. Equilibrium binding of Mn^{2+} to the brain GS currently is being measured using atomic absorption for the determination of Mn^{2+}.

Covalent labeling of nucleotide sites in brain GS was unsuccessful using the ATP analog, 5'-p-fluorosulfonylbenzoyladenosine. Some success has been obtained with the 2',3'-dialdehyde derivative of ATP (o-ATP) which is a substrate of the enzyme, and can be reduced with sodium borohydride. That is, a cyanogen bromide fragment having a strongly absorbing chromophore at 259 nm was obtained from the enzyme after treatment with a o-ATP but not from untreated enzyme. However, the specificity of the attachment of o-ATP is uncertain. Other analogs of ATP will be prepared by attaching 1,4-dibromo-2,3-butanedione to 6- and 8-mercapto ATP and tested in their reactivity and specificity towards the brain GS.

Rabbit antibody (polyclonal) directed against bovine brain GS has been prepared for our studies on this protein.

A quantitative amino acid analysis of the bovine brain GS has been performed. The tryptophan content is high -- 8 trp residues per subunit of M_r = 45,000, which accounts for the high specific absorption coefficient at 280 nm of this protein (see above). A sedimentation coefficient of $s_{20,w}$ = 16.0 S has been measured, consistent with brain GS being a globular protein with a frictional coefficient (f/fo) of 1.2 and M_r = 360,000.

(4) Thermodynamics of ligand-protein interactions. (Investigators: M. B. Blackburn, S. H. Neece, and A. Ginsburg).

Our calorimetric measurements of last year have been suspended while a LKB microtitrator was installed into our batch-type LKB microcalorimeter. This has required a lot of patience over the last 8 months since the microtitrator has required modifications and eventual interfacing to a computer. Calibrations are currently underway.

(5) Aspartate transcarbamoylase from E. coli: Studies on the Zn^{2+} bonding domains. (Investigators: J. B. Hunt (The Catholic University of America, Washington, D.C.), S. H. Neece, and A. Ginsburg in collaboration with H. K. Schachman at the University of California, Berkeley).

Aspartate transcarbamoylase (ATCase) from E. coli contains 6 catalytic (c) chains and 6 regulatory (r) chains (c_6r_6); the 4 -SH groups of each r chain are involved in tetrahedral bonding of Zn^{2+} near the c:r contact region (Monaco et al.: Proc. Natl. Acad. Sci. U.S.A. 75, 5276, 1978). Mercurials dissociate ATCase (Gerhart and Schachman: Biochemistry 4, 1054, 1965). The release of Zn^{2+} from ATCase and from isolated r subunit, upon challenge by p-hydroxymercuriphenylsulfonic acid (PMPS), and the rebinding of Zn^{2+} by these proteins, upon displacement of PMPS with 2-mercaptoethanol, have been studied using the sensitive, high-affinity metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) at pH 7.

Stopped-flow and spectrophotometric measurements have been used to obtain kinetic and equilibrium data while gel electrophoresis, light scattering, and ultracentrifugation were used to monitor changes in protein structure. Purified ATCase has been generously supplied by Dr. H. K. Schachman.

The following properties of PAR have been determined: with excess PAR, a 2:1 dye:Zn^{2+} complex is formed with a large absorption change at 500 nm, pH 7.0 ($\Delta\varepsilon$ = 66 x 10^3 M^{-1} cm^{-1}); the effective affinity β' is ~ 10^{12} M^{-1} for forming PAR_2 Zn^{2+}. The association of Zn^{2+} with PAR was not rate-limiting in our studies; a second order k = 2 x 10^7 M^{-1} s^{-1} for forming PAR-Zn was measured. The release of Zn^{2+} from the PAR_2Zn^{2+} can be rate-limiting, but experiments are underway to avoid this problem. PAR is useful also for following dissociation and assembly reactions of ATCase since a yellow to orange color change occurs upon mercurial-promoted dissociation of ATCase as the released Zn^{2+} forms the PAR_2Zn^{2+} complex. Upon the addition of excess 2-mercaptoethanol to displace the mercurial from regulatory subunits, Zn^{2+} rebinds to the protein, and the color changes from orange to yellow. If catalytic subunits are present under the latter conditions, ATCase assembles after Zn^{2+} rebinds to r chains. (Gel electrophoresis has been used to correlate visible spectral changes in PAR solutions with dissociation and assembly of ATCase.)

Studies on the mercurial-promoted release of Zn^{2+} from ATCase have been completed and can be summarized as follows: when the -SH group of each catalytic (c) chain is protected, 1 Zn^{2+} is released for every 4 eq of PMPS added to ATCase during titration of the 24 -SH groups of regulatory (r) chains. Moreover, the release of Zn^{2+} is a linear function of PMPS added, indicating that the rate-limiting step in Zn^{2+} release is mercurial attack on the 1st of the 4 r-SH groups bonded tetrahedrally to Zn^{2+} in an r chain near c:r contacts. Dissociation of ATCase is linked to Zn^{2+} release and mercaptide formation; e.g., upon addition of 4 eq of PMPS to ATCase in Hepes buffer, 1/6th of ATCase is dissociated to c_3 and r_2 subunits at ~ 83% of the rate of Zn^{2+} release, with no accumulation of the c_6r_4 intermediate as is observed in K-PO_4 buffer. Up to 4 PMPS/ATCase the release of Zn^{2+} is first-order in [PMPS] and is virtually independent of [ATCase] with an activation energy of 18 kcal/mol. With large excesses of PMPS, stopped-flow traces show a lag period followed by pseudo-first-order release of Zn^{2+} from ATCase and the reaction order in [PMPS] = ~ 1.3. Under these conditions, PMPS has a chaotropic effect on ATCase; the activation energy for Zn^{2+} release is much lower than that obtained with limiting PMPS and is increased by the presence of phosphate or active-site ligand from 6.6 to ~ 12 kcal/mol. Also, the enhancement in sulfhydryl-group reactivity promoted by active-site ligand binding to ATCase was less in Hepes than in K-PO_4 buffer. Inhibition of Zn^{2+} release by mercurial binding of buffer components was observed, and a participation of nonthiol protein groups of ATCase in mercurial binding is suggested by kinetic data.

The various observations from the 2 kinetic conditions presented (i.e., limiting [PMPS] and limiting [ATCase-SH] can be accommodated to a scheme (reactions 1 and 2) in which R-HgOH (the hydroxyl complex of PMPS) forms complexes with nonthiol amino acid side chains (ATCase-NH_3^+) in a pre-equilibrium step which serves to generate a species more susceptible to thiol attack.

$$1.\quad R\text{-}HgOH + ATCase\text{-}NH_3^+ \underset{}{\overset{K}{\rightleftharpoons}} R\text{-}Hg\text{-}NH_2\text{-}ATCase + H_2O$$

$$2.\quad R\text{-}HgOH + ATCase\text{-}SH \xrightarrow{k_2} R\text{-}Hg\text{-}S\text{-}ATCase + H_2O$$

Under conditions of limiting [PMPS] (E_a = 18 kcal/mol), increasing [ATCase] will tend to increase the rate of (2) directly but to decrease the rate of (2) indirectly by lowering [R-HgOH]. These two effects could nearly cancel, leading to an apparent nondependence on [ATCase].

$$\frac{d\ \ln\ [R\text{-}HgOH]}{dt} = \frac{k_2}{K}\ \frac{[ATCase\text{-}SH]}{[ATACase\text{-}NH_3^+]}$$

Under conditions of excess [PMPS], the binding of R-HgOH to nonthiol groups of ATCase in (1) could accelerate the reaction of PMPS with thiol groups in (2) through a chaotropic effect on ATCase which lowers E_a. The observed lag phase in stopped-flow measurements represents a steady state formation of a species more susceptible to thiol attack. In this case, the reaction order with respect to [PMPS] is greater than one.

$$\frac{d\ \ln\ [ATCase\text{-}SH]}{dt} = \frac{k_1\ [R\text{-}HgOH]^2}{k_{-1}/k_2 + [R\text{-}HgOH]}$$

Studies of Zn^{2+} release and rebinding to isolated regulatory dimers are in progress. Mercurial-promoted Zn^{2+} release is > 3000-fold faster from r_2 subunits than from ATCase. The rebinding of Zn^{2+} to r_2 subunits has been monitored by PAR but the dissociation of Zn^{2+} from PAR was rate-limiting. Also, the PAR-Zn^{2+} complex was found to deliver Zn^{2+} to r_2 subunits. Experiments are underway to separate these kinetic steps in the binding of Zn^{2+} to r_2 subunits.

Significance to Biomedical Research: The regulation and control of enzymic activities *in vivo* is of fundamental importance in cellular metabolism. *In vitro* studies of conformation and stabilization changes of biologically important protein macromolecules promoted by the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation are important in understanding cellular processes on a molecular basis.

Proposed Course:

(1) To study conformational and stabilization changes of protein macromolecules effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, calorimetry, spectral, viscometry, fluorescence, equilibrium binding, pH, electrophoretic, and kinetic techniques will be used.

(2) To study mutual interactions of divalent cations, substrates (or substrate analogs), and inhibitors with GS from *E. coli*. To locate specific sites in the subunit and the dodecameric structures in order to better understand tertiary and quaternary structural changes that occur on ligand interactions with GS.

(3) To characterize the reversible thermal transition of E. coli GS (discovered by A. Shrake while in this laboratory) more fully in terms of kinetic parameters. Also, the nature of the temperature-induced unfolding reaction (local vs temperature-induced macromolecular shape changes) will be investigated by viscometry, ultracentrifugation, and CD techniques.

(4) Studies on active-site ligand interactions with mammalian GS will continue. For this purpose, GS from bovine brain has been purified.

(5) Studies on the removal of Zn^{2+} from isolated regulatory subunits of E. coli aspartate transcarbamoylase (ATCase) and on the rebinding of Zn^{2+} to regulatory subunits will continue in order to better understand the mechanism of assembly of ATCase from catalytic and regulatory chains.

Publications:

Pinkofsky, H. B., Ginsburg, A., Reardon, I., and Heinrikson, R. L.: Lysyl residue 47 is near the subunit ATP-binding site of glutamine synthetase from Escherichia coli. J. Biol. Chem. 259: in press.

Ginsburg, A.: Book review, Methods in Enzymol. Vol. 99, Hormone Action. Anal. Biochem. 138: 265, 1984.

Maurizi, M. R. and Ginsburg, A.: Active-site ligand binding and subunit interactions in glutamine synthetase from Escherichia coli. Curr. Top. Cell. Regul., in press.

Hunt, J. B., Neece, S. H., Schachman, H. K., and Ginsburg, A.: Mercurial-promoted Zn^{2+} release from Escherichia coli aspartate transcarbamoylase. J. Biol. Chem. 259: in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00205-29 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Occurrence and Biochemical Roles of Selenium in Selenoproteins and seleno-tRNAs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Thressa C. Stadtman, Section Head, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS (if any)

Dr. Raymond Burk, Univ. of Texas Health Science Center, San Antonio, Texas

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Clostridial Glycine Reductase is an enzyme complex comprised of three protein components: selenoprotein A, a heat stable 12,000 M.W. protein that contains a selenocysteine residue; protein B, a protein of ca. 200,000 M.W. that contains essential carbonyl groups and protein C, ca. 250,000 M.W. Clostridium sticklandii and Clostridium purinolyticum, a purine fermenting organism, were used as enzyme sources. Pure 75Se-labled selenoprotein A preparations were isolated from both of these organisms. Small differences in acidity and primary amino acid compositions between the two protein A preparations were observed but the amounts of selenocysteine and cysteine were identical. Sequence analysis of peptides generated from the selenoproteins from these two sources show that the selenocysteine residues are internal and near the carboxy termini. The phases of this work already completed are basic to eventual analysis of the DNA that specifies the amino acid composition of this small selenoprotein and thus establish whether selenocysteine is the result of a post-translational modification of an amino acid specified by the genetic code. To date the mechanism of insertion of an essential selenocysteine residue in a variety of selenoproteins is completely unknown. The final purification by methods amenable to large scale work of the other two glycine reductase protein components has progressed and is now in final stages of development.

Seleno-tRNAs isolated from C. sticklandii and also from a methane producing organism, Methanococcus vanielii, were analyzed for amino acid acceptance activities and also for selenonucleoside contents. In addition to the already identified selenonucleoside, 5-methylaminomethyl-2-selenouridine, two related but different selenonucleosides were detected.

A project having to do with the occurrence of selenomethionine in two enzymes present in clostridium kluyveri was finished for the present time since this selenoamino acid was found to be inserted in the proteins as a non-specific substitute for the numerous methionine residues present in the enzymes, thiolase and beta-hydroxybutyryl-coenzyme A dehydrogenase.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives:

1. Anaerobic metabolism of certain amino acids and nitrogen heterocyles with special reference to the roles of selenium, molybdenum, quinones, flavins, and non-heme iron proteins in the electron transfer and phosphorylation processes involved.

a. Purification of the membrane associated proteins (proteins B and C) of Clostridial glycine reductase as a complex and resolution of the individual proteins from the complex. Characterization of proteins B and C. Mode of interaction of these proteins with the heat stable selenoprotein component of glycine reductase and studies on the mechanism of the catalytic reaction and the coupled phosphorylation of ADP.

b. Purification and characterization of the selenium-containing moiety of nicotinic acid hydroxylase from Clostridium barkeri and of xanthine dehydrogenase from Clostridium purinolyticum.

2. Isolation and characterization of selenoenzymes and selenium-containing tRNAs.

a. Structural studies on the selenoprotein A of glycine reductase and DNA-nucleotide sequence of the portion of the gene encoding the selenocysteine containing peptide in order to identify the amino acid precursor of the selenocysteine residue.

b. To determine whether or not the unidentified selenium component of two bacterial selenoenzymes (nicotinic acid hydroxylase and xanthine dehydrogenase) is part of the molybdopterin cofactor present in both enzymes.

c. Identification of seleno-tRNAs and distribution among bacteria and higher organisms. Biochemical roles of selenium-modified bases in tRNAs and mechanism of their biosynthesis.

Major Findings

1. The project to purify all three of the protein components of clostridial glycine reductase in amounts sufficient for detailed studies on enzyme reaction mechanism has been continued. Emphasis on development of methodology for isolatio of the C protein component in homogeneous form was required since this protein has not been purified completely in previous studies. Clostridium purinolyticum was cultured and used as an alternate source of the enzyme complex in a number of the studies. Although more difficult to culture reproducibly, this organism can be grown in a relatively defined medium with glycine as the major fermentable substrate and highly active glycine reductase is isolated from the cells. The selenoprotein A component isolated from this organism differs in some respects from the comparable protein produced by C. sticklandii, but it is similar in that it contains selenium as a selenocysteine residue and 2 cysteine residues are present. A simplified procedure for isolation of pure selenoprotein A from both organisms together with details of physicochemical properties of the protein from C. purinolyticum are described in the report of M. Sliwkowski. Partial amino

acid sequence data for the C. sticklandii protein have been obtained.

A comparison of the biological activities of the two selenoproteins showed that they differ considerably in specificity. For optimal reconstitution of glycine reductase activity when the B and C protein components are derived from C. sticklandii it is necessary to add the homologous selenoprotein A component; the C. purinolyticum protein A, which differs shomewhat in amino acid composition and in pK, is much less efficient. However both protein A species complement the B and C proteins from C. purinolyticum with similar efficiency. Tryptic peptides that contain the selenocysteine residue differ in size and amino acid composition when isolated from the two selenoprotein A species.

2. A project dealing with two seleno-enzymes from Clostridium kluyveri was completed for the time being. These enzymes, thiolase and β-hydroxybutyryl-CoA dehydrogenase, which were studied in collaboration with Dr. Maris Hartmanis (a visiting Swedish investigator) and Dr. Mark Sliwkowski, where shown to contain selenium in the form of selenomethionine. Although incorporation of selenium in these proteins appeared to be specific and independent of sulfur levels when the source of selenium in the growth medium was selenite, nevertheless the distribution of selenomethionine was random and mimiced methionine. This was demonstrated conclusively by elegant peptide analyses carried out by Dr. Sliwkowski on ^{35}S and on ^{75}Se labeled enzymes. The high methionine contents of the two enzymes together with the active sulfur and selenium metabolic systems in C. kluyveri appear to account for the conspicuous and readily detectable amounts of selenomethionine found. A number of growth experiments carried out with C. kluyveri showed that only limited substitution of selenium for sulfur could be tolerated. For example lowering of sulfur levels from 1 or 2 mM to 10 μM with concomitant elevation of selenium from 0.5 μM to 10 or 20 μM markedly inhibited growth. Hence the effects of more extensive substitution of selenomethionine in thiolase would seem difficult to achieve. In contrast to the marked ability of C. kluyveri to synthesize selenomethionine and incorporate it into proteins, no substitution of selenocysteine for cysteine was detected. This is important because the two proteins studied are also rich in cysteine residues. The claims in the nutritional literature of non-specific substitution of selenocysteine for cysteine in cereal grains may be suspect but valid analyses are lacking.

3. A collaborative project carried out with Dr. Raymond Burk of San Antonio, Texas has as its aim to purify and identify the 75,000 M_r selenoprotein produced by rats and found in liver and serum. The ^{75}Se-labeled protein, prepared by Burk's group is sent to us for development of additional purification procedures. High performance liquid chromatographic procedures using DEAE columns in addition to phenyl-separose chromatography and pyridoxal-phosphate affinity chromatography have been developed. These aspects of the work are carried out by Mr. J. N. Davis. The selenium in this protein was shown to be selenocysteine.

Proposed Course

(1) To define the precise role of selenium (in the form of a selenocysteine residue in the selenoprotein A component of clostridial glycine reductase) in the reductive deamination of glycine. It is known that the reduced selenoprotein serves as the immediate reductant in the reaction but the possibility that it also is directly involved in the concomitant phosphate esterification process

and ATP synthesis is still speculative. For this and other reasons studies are planned using substrate levels of glycine reductase reconstituted from pure preparations of the three constituent proteins. Using ^{35}S-labeled thiophosphate in place of orthophosphate the formation of ^{35}S-labeled adenosine-3-S-triphosphate will be studied. Attempts will be made to detect an enzyme-bound ^{35}S-labeled thiophosphate intermediate that may be present when ADP is not provided as final acceptor.

(2) To continue studies on the mechanism of specific incorporation of selenocysteine in proteins. Synthesis of DNA fragments corresponding to the sequence of amino acids around the selenocysteine residues in small peptides from the two species of seleno-protein A from C. sticklandii and C. purinolyticum will be undertaken. Currently this type of methodology is now relatively commonplace. This DNA could serve as probe for the complimentary messenger RNA. Antibodies to the native selenoprotein will be used to detect the in vitro translation product of this mRNA. If correct, the complete DNA coding for the selenoprotein could then be obtained for sequence analysis. The triplet specifying the amino acid in the position of selenocysteine would identify the primary translation product.

Shot gun attempts to clone the DNA for selenoprotein A may also be tried in case larger amounts of DNA and mRNA are needed for this type of approach. Antibodies to protein A were originally produced on a small scale in a rabbit but sheep will now be used.

(3) Collaborative work with Dr. Raymond Burk on the unidentified selenoprotein produced by rats (synthesized in liver and exported to serum) to isolate in pure form for:

(a) identification of catalytic function and
(b) use as antigen for preparation of specific antibodies will be continued

Honors

Invited Plenary Lecturer- 4th International Conference on Organic Chemistry of Selenium and Tellurim; Birmingham, England, July 1983.

Member of Biochemistry Delegation to Peoples Replublic of China, Sept. 1983.

Invited Plenary Lecturer- 3rd International Symposium on Selenium in Biology and Medicine; Beijing, China, June 1984.

Invited Plenary Lecturer- 11th International Symposium on the Organic Chemistry of Sulphur; Lindau, Germany, Sept. 1984.

Invited Speaker- Gordon Conference on Methanogenesis; Tilton, N.H. August 198-

Publications

Stadtman, T. C.: Some vitamin B_{12} and Selenium-dependent enzymes. In An era in New York Biochemistry: A Festchrift for Sarah Ratner. New York Acad. Sci. Series II 41, 233-236 (1983).

Stadtman, T. C.: New biologic functions-selenium-dependent Nucleic Acids and Proteins. Fund. Appl. Toxicol. 3, 420-423, 1983.

Ching, W.-M., Wittwer, A. J., Tsai, L. and Stadtman, T. C.: Distribution of two selenonucleosides among the selenium-containing tRNAs from Methanococcus vannieli. Proc. Natl. Acad. Sci. USA 81: 57-60, 1984.

Stadtman, T. C., Ching, W.-M., Hartmanis, M., Sliwkowski, M., Tsai, L., Wittwer, A. J., and Yamazaki, S.: Bacterial selenoenzymes and seleno-tRNAs: Studies on chemical composition and structure. In Berry, F. J. and McWhinnie (Eds.): Proceedings of the 4th International Conference on Organic Chemistry of Selenium and Tellurium. Birmingham, England, Univ. of Aston, 1983, pp. 521-530.

Wittwer, A. J., Tsai, L., Ching, W.-M. and Stadtman, T. C.: Identification and synthesis of a naturally occurring selenonucleoside in bacterial tRNAs: 5-Methylamino-methyl-2-selenouridine. Biochemistry, 1984, in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00206-25 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stereochemical Studies of Enzymatic Reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, Title, Laboratory, and Institute)

PI: Lin Tsai, Research Chemist, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS (if any)

None

LAB BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

5-Aminomethyl-2-selenouracil was synthensized. Its identitiy with the 75-Se-selenopyrimidine base from the 75-Se-selenonucleoside produced by a E. coli mutant suggested that the new selenonucleoside was 5-aminomethyl-2-selenouridine.

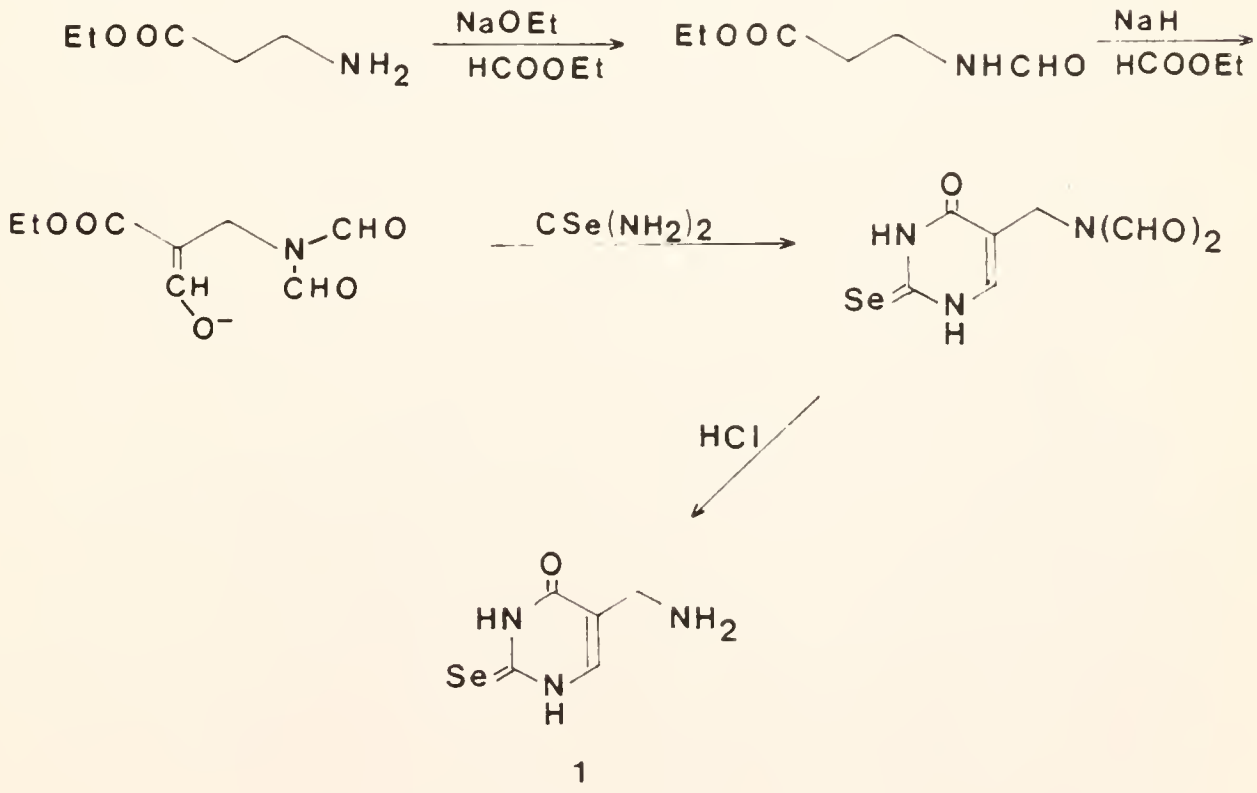
45

Project Description:

Objectives: Since the novel selenium-modified nucleoside in the tRNA of E. coli was established to be 5-methylaminomethyl-2-selenouridine. Dr. A. J. Wittwer showed that this selenonucleoside was absent in the tRNA of a mutant, deficient in the production of 5-methylaminomethyl-2-thiouridine. Instead, a different selenonucleoside was observed in the HPLC analysis of the hydrolysate of the tRNA. Therefore, it is of considerable interest to determine the structure of this new selenonucleoside from the mutant.

Major Findings:

The new selenonucleoside from the mutant had an UV spectrum similar to that of a 2-selenouridine derivative. It could be converted to 5-methylaminomethyl-2-selenouridine by an in vitro methylating system. It was, therefore, tentatively assigned the structure, 5-aminomethyl-2-selenouridine. In order to confirm this assignment of structure, a synthesis of 5-aminomethyl-2-selenouridine was undertaken as shown in Scheme 1.



1

Although the synthesis was designed according to the method successful for the synthesis of the corresponding N-methyl compound, unexpected difficulties were encountered. Consequently very poor yield of the desired product was obtained. However, the product did exhibit UV and mass spectral properties consistent with the assigned structure (1). Furthermore, a sample of the

synthetic 5-aminomethyl-2-selenouracil (1) was shown by HPLC analysis to be identical with the ^{75}Se-selenopyrimidine base obtained by dilute hydrochloric acid treatment of the ^{75}Se-selenocleoside isolated from the mutant. From this observation, one can deduce that the new selenonucleoside produced by the mutant is most likely 5-aminomethyl-2-selenouridine.

For comparison, 5-aminomethyl-2-thiouracil was also synthesized by this method.

Proposed Course of Action:

To improve the synthesis of 5-aminomethyl-2-selenouracil and to design snythetic methods for analogous selenonucleosides.

Publications:

Ching, W.-M., Wittwer, A. J., Tsai, L. and Stadtman, T. C.: Distribution of two selenonucleosides among the selenium-containing tRNAs from Methanococcus vannielii. Proc. Natl. Acad. Sci. U.S.A, 18, 57-60 (1984).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00211-11 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Protein Oxidation in Protein Turnover and in Aging

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

PI:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB, NHLBI
Others:	M. E. Wittenberger	Biological Laboratory Technician	LB, NHLBI

COOPERATING UNITS (if any)

None

LAB BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.4	1.1	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Other workers have shown that the glucose-6-phosphate dehydrogenase (G6PDH) in "old" animals and in cultured fibroblasts of humans with accelerated aging diseases (progeria, Werner's syndrome) is less active and is more sensitive to heat inactivation than is the G6PDH in young animals or in cultured cells from normal individuals. Results of the present study show that the exposure of pure G6PDH from bovine adrenals to a mixed-function oxidation system comprised of ascorbate, Fe(II) and O_2 leads to inactivation of the enzyme and to the oxidation of some amino acid residues to carbonyl derivatives. The substrates NADPH and glucose-6-P protect the enzyme from this inactivation. The residual activity that remains after only 50% of the G6PDH has been inactivated is much less stable to heat inactivation than is a control sample that had been incubated in the absence of ascorbate and Fe(II). The results demonstrate that previously observed age-related changes in G6PDH activity might be caused by MFO mediated oxygen radical damage. It was incidentally discovered that G6PDH is a "cold-sensitive" enzyme. At 4°C, the enzyme dissociates to less active subunits. This inactivation can be reversed by raising the temperature to 37°C. The cold inactivation is prevented by the presence of NADPH but is accelerated by glucose-6-P.

The amino acid residue in enzymes that is oxidized to a carbonyl derivative by MFO systems has not been identified. Studies with amino acid homopolymers show that a MFO system comprised of ascorbate, Fe(III), EDTA and O_2 catalyze the generation of carbonyl groups in polylysine, polyhistidine, polyarginine, polyproline, and polyserine. Thus, any one of several amino acids can be a target for MFO catalyzed oxidation.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: Previous studies in this laboratory have shown that many enzymes are inactivated by any of several different mixed-function oxidation (MFO) systems and that following such inactivation these enzymes become good substrates for highly specialized proteases present in bacteria and mouse liver cytosol. Many of the enzymes that are susceptible to oxidative inactivation by MFO systems have been shown by other workers to accumulate as inactive or less active forms during aging. This suggests that some age-related changes in enzyme activity could be due to MFO-catalyzed oxidative reactions. Glucose-6-phosphate (G6PDH) is one of the enzymes previously shown to undergo age-related loss of activity. Moreover, the "old" enzyme is more susceptible to heat denaturation than is the "young" enzyme. It therefore follows that if the age-related changes are provoked by MFO systems, exposure of active enzyme to a MFO system should lead to progressive loss of catalytic function and to an increase in heat stability of the residual enzyme activity. The present study was undertaken to investigate these possibilities.

Major Findings:

It was found that the G6PDH from bovine adrenals is readily inactivated by incubation with an MFO system comprised of ascorbate, EDTA, $FeSO_4$, and O_2. The residual activity that remained after 50% of the initial activity was lost was very much more sensitive to heat denaturation. Preliminary studies (with J. Rivett) show that although the oxidized G6PDH is not degraded by the neutral protease, from rat liver cytosol, it is more effective than the native enzyme in inhibiting the ability of the protease to degrade oxidized glutamine synthetase. This suggests that the oxidized G6PDH can bind to the neutral protease with higher affinity than does native G6PDH. Substrates of the enzyme, glucose-6-phosphate and NADH, protect the G6PDH from oxidative inactivation.

It was incidentally noted that the G6PDH from bovine adrenals is a "cold sensitive" enzyme. During incubation at 0°C, the tetrameric enzyme undergoes subunit dissociation to less active monomers. Upon raising the temperature to 37°C, the enzyme undergoes reassociation to the tetrameric form. The temperature-dependent dissociation of G6PDH is inhibited by the substrate NADPH but is stimulated by the substrate glucose-6-phosphate.

The amino acid residue in enzymes that is oxidized to a carbonyl derivative by MFO systems has not been identified. Studies with amino acid homopolymers show that a MFO system comprised of ascorbate, Fe(III), EDTA and O_2 catalyze the generation of carbonyl groups in polylysine, polyhistidine, polyarginine, polyproline, and polyserine. Thus, any one of several amino acids can be a target for MFO catalyzed oxidation.

Significance to Biomedical Research and Program of the Institute:

The demonstration that the MFO-catalyzed oxidation of G6PDH leads to the formation of inactive and less active heat-sensitive forms supports the view that the alteration of enzymes that occurs during aging is due, at least in part, to oxidative damage. This suggests that during aging, one or more of three events occurs: (1) the enzymes become more sensitive to oxidative attack; this could

occur, for example, if the levels of enzyme substrates are reduced or if the levels of oxygen radical scavengers such as catalase, superoxide dismutase, glutathionine peroxidase, vitamin E, citrate, etc. are lowered: (2) the levels of mixed-function oxidation systems (viz, cytochrome P_{450} reductase, NAD(P)H oxidase, nonheme iron proteins, peroxidase) increase; (3) the levels of proteases that degrade the altered enzyme forms decrease.

Proposed Course:

Studies similar to those described here for G6PDH will be made on other enzymes known to accumulate during aging. The amino acid residues that are modified by MFO systems will be identified. Efforts will be made to evaluate the contribution of the three factors mentioned that might be responsible for the accumulation of altered enzymes during aging. Efforts will be made to develop in vivo models to explore the roles of enzyme oxidation in aging.

Publications:

Nakamura, K. and Stadtman, E. R.: Oxidative inactivation of glutamine synthetase. Proc. Nat. Acad. Sci. U.S.A. 81: 2011-2015, 1984.

Oliver, C. N., Ahn, B., Wittenberger, M. E., and Stadtman, E. R.: Oxidative inactivation of enzymes: Implication in protein turnover and aging. In Ebashi, S. (Ed.): Cellular Regulation and Malignant Growth. New York, Academic Press, 1984, in press.

Rivett, A. J., Roseman, J. E., Oliver, C. N., Levine, R. L., and Stadtman, E. R.: Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. In Khairallah, E. A., Bond, J. S., and Bird, J. W. C. (Eds.): Intracellular Protein Catabolism. New York, Alan R. Liss, 1984, in press.

Oliver, C. N., Fulks, R., Levine, R. L., Fucci, L., Rivett, A. J., Roseman, J. E., and Stadtman, E. R.: Oxidative inactivation of key metabolic enzymes during aging. In Roy, A. K. and Chatterjee, B. (Eds.): Molecular Basis of Aging. New York, Academic Press, 1984, pp. 237-264.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00212-13 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Ammonia-Assimilatory Enzymes in E. coli K12

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

PI:	Mary Anne Berberich	Research Chemist	LB, NHLBI
Others:	Sue Goo Rhee	Research Chemist	LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Genetic and biochemical studies with enterobacteria reveal that nitrogen control in these organisms is very complex. Nitrogen control can be defined as the enzymatic adjustment made by cells in response to the availability of nitrogen supplied as NH_4 salts or amino acids in the growth medium. In general, when the nitrogen supply is limiting, the synthesis of the ammonia assimilatory enzymes, some amino acid transport systems, and some amino acid catabolic enzymes increases. This aspect of nitrogen regulation is under the control of at least three regulatory loci. The function of the products of the genes glnF, glnG, glnL has yet to be determined, although both positive and negative control mechanisms appear to be involved.

The study of the positive aspects of nitrogen control under conditions saturating for negative control has been a principal focus of this laboratory. The results of these studies show that nitrogen control reflects the catabolic capacity of the cell and that utilizable nitrogen sources and some carbon sources are, to some extent, in competition for this capacity.

Other studies have focused on the possibility that an additional regulatory role in nitrogen control might exist for some of the proteins which participate in the cascade for modulation of the enzyme activity of glutamine synthetase (GS). The evidence so far indicates an interaction between the elements of the activity modulation cascade and certain genetic elements involved in the regulation of synthesis.

In order to clarify the relationship between the non-glnG/L, non-glnB type of constitutivity and adenyltransferase which was noted in some earlier work, genetic experiments to assign map position to mutations affecting these functions have continued.

Project Description:

Objectives:

1. To explore the hierarchy of nitrogen control in E. coli K12.

   a. determine physiological effectors of nitrogen control.
   b. determine response of regulatory mutants to physiological effectors of nitrogen control.
   c. identify other enzymatic components involved in positive activation by D-amino acids.

2. To study the interactions between the elements of the modification cascade which regulates GS activity and the genetic elements which have a role in regulating GS synthesis.

3. To conduct genetic experiments for the purpose of mapping mutations involved in nitrogen control.

   a. assign genetic location to structural gene for adenylyl-transferase.
   b. locate the non-GL, non-glnB constitutive mutations.
   c. isolate glnF isogenic with the other mutant strains derived in this laboratory.
   d. construct gln-lac fusion strains for the purpose of (i) localizing regulatory components in cells; (ii) studying their regulation.

Methods Employed:

1. The Hierarchy of Nitrogen Control in E. coli K12 N99.

a. Physiological effectors of nitrogen control. Table I summarizes data which shows that addition of D-glutamate, D-lysine, D-threonine or glycine to cells growing in a glycerol-mineral salts-high ammonia medium provokes an increase in the level of GS. The increase in GS level observed upon addition of the Damino acids appears to be additive. At saturating concentrations of D-glutamate, addition of D-threonine and D-lysine further increase the GS level by 128%. The effect of D-glutamate addition is independent of mutations in gltB (GAT), gdh (GDH), or glnA (GS). Not shown are the data which indicate that the increases in GAT and GDH are specifically provoked by D-glutamate. Addition of D-lysine, D-threonine, or glycine does not effect an increase in the levels of these enzymes.

That the level of GS in E. coli is responsive to the carbon source as well as the nitrogen source supplied in the growth medium is also shown in Table I. When cells are grown on lactose-mineral salts-high ammonia medium, the specific activity of GS is at the physiologically derepressed level. As with cells grown on glycerol or glucose carbon, addition of glutamine to the lactose medium results in approximately 30% lower GS level. Addition of L-glutamine simultaneously with D-glutamate decreases the positive activation effect provoked by this compound by 50%.

TABLE I. Catabolic Effectors of GS Level in E. coli

		Carbon Source[a]					
		glycerol		glycerol + D-glutamate[b]		lactose	
Relevant genotype	function affected	GS (units/mg)	$\bar{n}$	GS (units/mg)	$\bar{n}$	GS (units/mg)	$\bar{n}$
wild type		0.27	2.6	0.49	1.6	1.1	10.3
				0.41[c]	7.6		
				0.42[d]	5.4		
				0.43[e]	5.8		
				0.61[f]	4.0		
				1.04[g]	1.0		
glnD	UT	0.02	10.4	0.3	10.3	ND	--
glnG	regulation	1.37	7.1	1.61	7.7	2.3	11.4
glnD glnC	regulation	1.54	10.7	1.40	10.1	1.9	11.1
glnB	P_{II}	0.88	8.9	0.87	8.3	2.0	11.0
glnF[h]	regulation	NM	--	NM	--	NA	--
glnD glnE	UT, AT	0.15	0	0.21	0	0.25	0.2
O/P glnA	regulation	0.36	1.8	0.33	0.7	0.56	9.1

[a]Medium = mineral salts with NH_4Cl at 100 mM.

[b]D-glutamate was added at 10 mM to log phase cells 1 h prior to harvesting cells at Klett 100.

[c]D-lysine as in b.

[d]D-threonine as in b.

[e]Glycine as in b.

[f]D-glutamate + D-lysine + D-threonine + glycine as in b at 2.5 mM each (total conc = 10 mM).

[g]D-glutamate + D-lysine + D-threonine as in b at 10 mM each.

[h]S. typhimurium LT_2 (SK100).

NM no measurable activity.

NA not appplicable.

ND not determined.

b. Response of regulatory mutants. Further studies with strains carrying mutations in nitrogen regulatory genes showed that the response to D-glutamate addition depends on a functional glnG gene (Table I). Strains containing mutations which interfere with positive activation of glnG also fail to demonstrate a positive response to D-glutamate addition. The data obtained with lactose-grown cells suggest that regulation of GS level reflects a bimodal control mechanism. From studies with mutants in regulatory genes, it would appear that glnG mediates both affects and that P_{II} exerts its effect primarily via the nitrogen regulatory mode. The response of o/p glnA, which is refractory to the nitrogen regulatory mode but responsive to carbon effects, supports the bimodal control hypothesis.

Other data indicate that the levels of GDH and GAT are affected by, though not strictly dependent upon, the regulatory elements involved in nitrogen control. The observation that GlnF mutants have GDH and GAT levels ~ 60% that of comparable wild type while glnB mutants demonstrate elevated GDH levels in the absence of D-glutamate supports this conclusion.

c. Identification of other enzymatic components involved in positive activation by D-amino acids. Consistent with the idea that D-glutamate may be a physiological effector of nitrogen control is the finding that glutamate racemase (GR) activity is elevated in extracts of cells grown under nitrogen derepressing conditions (Table II). When D-glutamate is added to cells growing under nitrogen sufficient conditions, the specific activity of glutamate racemase is increased by 39% (Table III). That this increase represents enzyme activation is shown by the results obtained when D-glutamate and chloramphenicol were added simultaneously. Whereas the increases in GS, GDH, and GAT could not be elicited by D-glutamate in the presence of chloramphenicol, the glutamate racemase appeared to be further activated an additional 52% after 60 minutes exposure. In addition, a flavoprotein glutamate dehydrogenase activity was demonstrated in cell-free extracts and was at least 30% elevated when D-glutamate had been added to the growth medium. This enzyme was active at pH 6.5 and demonstrated a 7-fold greater activity vs L-glutamate as compared with the D-isomer. This activity may reflect a coupling with the glutamate racemase since it is also present in the extracts.

TABLE II. Glutamate Derepressing Conditions and Glutamate Racemase Activity in Wild Type E. coli

Nitrogen source in growth medium	Enzyme activity (units/mg)			
	GR•	GS•	GAT•	GDH•
Glutamate[a]	0.829	2.03	0.08	0.50
Glutamate + NH_4Cl[a]	0.332	0.34	0.08	0.34

•Specific activities determined in cell-free extracts.
[a]Glutamate concentration was 60 mM; NH_4Cl concentration was 40 mM.

TABLE III. Dependence of D-glutamate Provoked Responses on Protein Synthesis in Wild Type

Additions••	Enzyme activity (units/mg)						
	GS	n̄	GAT	GDH	GR	GO•	
						L	D
None	0.332	3.8	0.259	0.549	0.44	3.37	0.512
D-glutamate	0.534	2.0	0.411	0.979	0.61	4.38	0.377
D-glutamate + cm	0.352	4.2	0.290	0.476	0.93	N.D.	N.D.

•GO = glutamate oxidase activity vs L-glutamate and D-glutamate.
••D = glutamate was added at a final concentration of 10 mM to cells growing in glycerol-NH_4Cl. Chloramphenicol was added simultaneously with D-glutamate to a final concentration of 10 μg/ml.

2. Interactions between Mutations Affecting the Modification Cascade and the Regulatory Process.

While mutations in glnB (P_{II} structural gene) result in constitutivity of GS at a high level of synthesis, mutations in glnD (UT/UR structural gene) result in glutamine bradytrophy due to the fact that they lead to the production of extremel low levels of highly adenylylated GS. In the case of glnD mutants, when glutamin

is supplied as the sole N source in the growth medium, an increase in the level of highly adenylylated GS approaching that of derepressed wild type can be observed as the concentration of glutamine in the medium is increased in the range of 10-30 mM. Therefore, positive activation is affected by mutations in glnD. The small increase observed for glnD mutants in response to D-glutamate addition agrees with this interpretation. We had previously determined that extracts of a glnD, glnB double mutant had P_{II} activity in an in vitro assay system. This would suggest that the phenotypic suppressor mutation encodes an altered protein which functions in vivo to release glnD (UT^-) mutants from glutamine dependency while imparting constitutive synthesis of GS. GlnB results in constitutive GS in a $glnD^+$ background also. Again, interaction between the modification cascade and regulation of synthesis is indicated.

We have observed that extracts of cells carrying mutations such as glnB (P_{II}) or gln"C" (non-GL, non-glnB) contain high levels of highly adenylylated GS under all conditions of growth. The data summarized in Table I indicate that for all strains examined, growth on lactose leads to increase in $\bar{n}$ as well as increase in GS level. On the other hand, when GS level is increased upon D-glutamate addition, no increase in $\bar{n}$ is observed. The smallest differential between D-glutamate addition and lactose growth on the level of GS is seen in the case of the AT^- and the "C" type mutants where $\Delta\bar{n}$ is minimal.

As with glucose-grown cells, addition of glutamine to cells growing in lactose-ammonia medium reduced the level of GS by approximately 30%. However, cells cultured on lactose-mineral salts with glutamine as sole nitrogen source (derepressing conditions) exhibited an exaggerated growth lag, taking approximately 48 hours to reach the O.D. where cells were usually harvested. At this point, the level of GS was 2 X that of the glucose-glutamine grown cells. Exaggerated growth lag on cultural downshift from glucose-ammonia-glutamine to glucose-glutamine is another consequence of the AT^- mutation which has been noted previously. These findings may indicate involvement of adenyltransferase in the mobilization of carbon and nitrogen sources.

3. Genetic Mapping of Mutations Involved in Nitrogen Control.

a. Adenyltransferase. Initial experiments using a dapE recipient (CGSC 4544) suggested a location for AT in this region. However, subsequent transductions using dapA (CGSC 4548) did not bear this out. It was discovered that strain 4544 contains a temperature-sensitive mutation which results in constitutivity of GS at 30°C (where the crosses were performed using Ø p1 CAM(AT^-). Using plvir(AT^-) and a dapA recipient, no transfer of AT^- could be observed in transductants to $dapA^+$. Likewise, no linkage could be observed by transduction with other markers in the area (nupC; purC; ptsI-cysA,K; Zfa-1::tn10) where AT^- was employed as either donor or recipient. Introduction of purC::tn10 into the strain carrying an AT^- mutation imposed an adenine requirement, allowing its use as recipient in Hfr crosses. Using Hfr KL16, which transfers counterclockwise from a point of origin at 61', and Hfr pK191, which transfers clockwise from a point of origin at 43', adenine-independent recombinants were selected. All of these remained AT^-, indicating that the structural gene for adenyltransferase is not located between 43 and 61 min on the E. coli chromosome.

Starting with plc18-28 from the collection of John Carbon, other workers in the laboratory are cloning the gene for adenyltransferase. In vivo, plC18-28 corrects the AT⁻ defect in strain MB1-7 and causes an increase in the level of GS as well. We could demonstrate complementation of ptsI-cysA,K with plc18-28 by standard genetic methods. Plc fragments are < 1 mm in size, so it was not surprising that no complementation was observed for dapA(4548), dapE(4544), dapAE (CBK110), purC(4900), purC::tn10(6187). In addition, plc18-28 appeared to inhibit a glnD (UT⁻) mutant, which was surprising since AT⁻ mutants were originally selected in this laboratory as phenotypic suppressors of the glnD imposed glutamine growth requirement. Using specific tester strains for amber suppression, it could be shown that plc18-28 also carries supN which would define its minimum span from 52-52.35 min.

One explanation for the failure to demonstrate a location for the structural gene for adenyltransferase in the region where complementation by plc18-28 is observed might be that the correction of the AT⁻ defect reflects a functional complementation at the biochemical level. A component of the phosphotransferase system might be implicated since subsequent findings reveal that carbon source is involved in the regulation of GS.

More recent experiments to locate glnE have been carried out by insertion of tn10 mutations into the AT⁻ strain, which yield a scoreable phenotype, and selecting for correction to prototrophy by Hfr transfer. After purification of recombinants, clones are grown and assayed for AT activity along with the AT⁻ derivative used in the cross, as well as all parents and the donor. So far, no position for AT can be assigned on the genetic map although ~ 50% of the chromosome has been examined.

b. Constitutive type UT suppressor. After determining that gln"C" was not linked to glnA,L,G, glnD, glnB, or glnF, it was determined that this type of constitutivity was suppressible by F100, a large episome spanning the gal operon. Growth on galactose was the selection employed for the maintainance of the episome since all derivatives of N99 are galK⁻. After examination of a series of overlapping deletions in this genetic region, it was concluded that the suppression by F100 was functional, i.e., due to growth on galactose. Physiological studies on carbon source effects subsequently showed this to be the case. Genetic mapping studies using the method described above have continued. Recently, this mutation has been localized in the genetic region between 43 and approximately 55 min. The region complemented by plc18-28 is included in this segment as is another component of the phosphotransferase system, ptsF. Experiments to determine a more precise location for gln"C" by co-transduction are in progress.

c. A glnF knock-out mutation isogenic with the derivatives of N99 described here is required. The method employed involved the preparation of a transducing pl tn10 pool using an E. coli strain carrying F'141. Since F'141 covers the glnF region, all glutamine auxotrophs selected by transduction to tetracycline resistance employing this lysate should be in glnF. A number of isolates were obtained in this way and are currently being characterized. However, sensitivity to glutamine complicates use of these derivatives.

d. A number of glutamine-minus strains have been constructed using a combination of mu phages which create gln-lac gene fusions. Work with these

isolates is also complicated by sensitivity to glutamine on glucose as well as on the lactose selection medium.

Proposed Course:

1. Continue to explore the hierarchy of nitrogen control.
   a. follow through on enzymatic evaluation of D-amino acid effects.
   b. study effect of growth on lactose in glnD mutants.
   c. assess lactose effects on GAT and CDH levels.

2. Continue studies on interaction between modification cascade and regulatory components. Assess recently constructed $glnD^+$, "C" and $glnD^+$, $glnE^-$ strains

3. Proceed with genetic analyses

   a. continue mapping experiments with AT^- and non-GL, non-glnB, gln"C" mutants.
   b. continue characterization of putative glnF::tn10 isolates.
   c. continue studies with ϕ gln-lac strains.

Publications:

Berberich, M. A.: Metabolic effectors of nitrogen control. Curr. Top. Cell. Regul., 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00224-07 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Calcium-regulated Protein Phosphatases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Charles Y. Huang	Research Chemist	LB, NHLBI
Others:	Marita M. King	Visiting Fellow (October, 1983-June, 1984)	LB, NHLBI
	May M. Cheung	Summer Student Assistant (June-August, 1984)	LB, NHLBI
	Kirk K. Lynn	Summer Student Volunteer (July-August, 1984)	

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.1	1.8	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues xx☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) Concomitant with activation by Ca^{2+} and calmodulin, the calmodulin-dependent phosphoprotein phosphatase was found to undergo a time-dependent deactivation process. The deactivation is a first-order reaction and is accelerated by the presence of substrate and competitive inhibitors. It can be reversed or prevented by addition of divalent metal ions like Ni^{2+}, Mn^{2+}, Mg^{2+}, etc., which have been shown previously to activate the enzymatic activity. This study indicates that the phosphatase is not simply a Ca^{2+}-calmodulin-stimulated enzyme. It requires at least one additional metal ion for structural stability.

(2) Examination of the mechanism of deactivation of the calmodulin-dependent protein phosphatase led to the discovery that the enzyme contains 0.2-0.6 mol of phosphate per mol of enzyme. It also contains nearly stoichiometric amounts of Fe^{3+} and Zn^{2+}.

(3) The minimum number of Ca^{2+} ions needed for the activation of calmodulin-dependent protein phosphatase was found to be 3.5 and 1.7 in the absence and presence of Mg^{2+}. The observation suggests that the presence of the activating metal ion enhances the affinity of the enzyme for calmodulin not saturated with Ca^{2+}.

(4) The activation of calmodulin-dependent protein phosphatase by Ni^{2+} ions is a time-dependent, first-order process. Like the deactivation reaction, the presence of substrate accelerates the activation. Interactions among the active-site, the metal ion binding, and the calmodulin binding domain are implicated.

(5) A Ca^{2+}-inhibited protein phosphatase has been partially purified from bovine brain. The enzyme catalyzes the dephosphorylation of two phosphorylated sites on synapsin I selectively. The results indicate that this phosphatase and the calmodulin-dependent phosphatase may function in vivo in a coordinated manner.

Project Description:

Objectives:

(1) To gain knowledge of regulatory and catalytic mechanisms of enzymes mediated by Ca^{2+}.

(2) To develop or improve methods and theories applicable to the study of enzymes.

Major Findings:

(1) Calmodulin-dependent deactivation of protein phosphatase and the requirement of a divalent metal ion other than Ca^{2+}. Concomitant with activation by Ca^{2+}-calmodulin (CaM), the CaM-dependent protein phosphatase (CDPP, also known as calcineurin) was found to undergo a time-dependent deactivation process. The deactivation required the presence of both Ca^{2+} and CaM, and the deactivated enzyme displayed little or no catalytic activity. The main features of the deactivation are as follows:

(a) The deactivation process followed first-order reaction, implicating a conformational rearrangement of the CaM-CDPP complex.

(b) The process was accelerated by the presence of the substrate p-nitrophenyl phosphate (PNPP) and by inhibitors such as PP_i and di- and triphosphate nucleotides. A theory has been developed to analyze the reaction. The inhibitors were found to be competitive with the substrate. Deactivation rate constants for E•CaM, E•CaM•PNPP, and $E•PP_i$, and E•CaM•ADP were found to be 0.06, 0.47, 2.3, and 8.0 min^{-1}, respectively. K_m and k_{cat} for CDPP in the presence of CaM were 11 mM and 46 min^{-1}, and K_i's for PP_i and ADP were 55 μM and 0.94 mM. Since preincubation of CDPP with PNPP or inhibitors in the absence of CaM had no effect, the accelerated deactivation by these compounds indicate that only E•CaM complex can undergo further ligand-induced conformational changes.

(c) That the deactivation reflects a conformational rearrangement of CDPP is supported by exclusion of other possibilities: (i) the deactivation is not due to severe product inhibition because it occurs during preincubation with Ca^{2+} and CaM alone; (ii) it is not due to dissociation of CaM since the deactivated phosphatase cannot be eluted from CaM-Sepharose column unless an EGTA-containing buffer is applied; (iii) it is not due to proteolysis since enzymatic activity can be regained by addition of divalent metal ions; (iv) loss of a crucial ion is ruled out since the metal ion content before and after deactivation remains unchanged. Furthermore, exhaustively dialyzed CDPP behaves just like the undialyzed enzyme when subjected to deactivation; (v) self-dephosphoryaltion is not the cause since the content of covalently attached phosphate is the same before and after deactivation.

(d) Deactivation cannot be reversed by the dissociation of CaM. Addition of EGTA followed by Ca^{2+} to effect dissociation and reassociation of the E•CaM complex has no effect. However, the deactivated enzyme can be readily reactivated by the addition of divalent metal ions such as Ni^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, and Zn^{2+}.

The deactivation study reveals that CDPP is not simply a Ca^{2+}-CaM-stimulated enzyme. It requires at least one additional metal ion for structural stability and full activity. Acceleration of deactivation by substrate and competitive inhibitors further indicates interactions among the active site, the metal ion binding site, and the enzyme-CaM binding domain. If a metal ion existing at very low levels in vivo like Ni^{2+} or Mn^{2+} ($\sim 10^{-7}$ M) is required, there may exist a metal carrier protein to transfer the ion or serve as a regulator. Otherwise, the metal requirement may be satisfied by the abundant Mg^{2+} ion.

(2) Identification of CDPP as a phosphorylated, Zn- and Fe-containing metalloenzyme. Examination of the mechanism of deactivation of CDPP led to the discovery that CDPP contains 0.2-0.6 mol of phosphate per mol of enzyme. The role and nature of phosphorylation of CDPP is not yet known. Atomic absorption experiments revealed that the phosphatase contains nearly stoichiometric amounts of nondialyzable Fe and Zn; Al is also present in smaller amounts. The Fe has been shown to exist primarily in the Fe^{3+} state. Removal of these ions requires a protein denaturant and a sulfhydryl reagent.

(3) Ca^{2+} activation of CDPP. To determine the number of Ca^{2+} ions involved in the activation of CDPP by CaM and the contribution of Ca^{2+} bound to the B subunit of CDPP, experiments similar to those performed with cAMP phosphodiesterase (Huang et al., Proc. Nat. Acad. Sci. U.S.A. 78, 871-874, 1981) were carried out in the absence and presence of Mg^{2+} ions. Preliminary calculations indicate that in the absence of an activating ion like Mg^{2+}, a minimum of 3.5 CaM-bound Ca^{2+} ions are required for activation, whereas in the presence of Mg^{2+}, 1.7 CaM-bound Ca^{2+} ions are required. The implications are: (a) Similar to cAMP phosphodiesterase, fully liganded CaM (4 Ca^{2+}) ions) may be the activating form in the absence of Mg^{2+}. (b) The presence of Mg^{2+} reduces the number of CaM-bound Ca^{2+} required for activation. The number of Ca^{2+} ions bound to the B subunit of CDPP that are involved in activation cannot be estimated since the Ca^{2+} binding constants for the B subunit have not been properly determined.

(4) Mechanisms of activation of CDPP by Ni^{2+} ions. Activation of CDPP by Ni^{2+} ions is a time-dependent process characterized by a lag time. Analysis of the lag reveals that it is a first-order process with a rate constant of ~ 2 min^{-1} at a PNPP concentration of 10 mM. Like the deactivation reaction, higher levels of PNPP accelerate the activation process. This finding further strengthens the notion that interaction between the active-site and the activating metal binding site results in a more rapid conformational rearrangement of the phosphatase. The Ni^{2+} ion is bound to the 60,000 molecular weight A subunit as evidenced by a Trp-fluorescence change on adding Ni^{2+} to the separated A subunit (prepared by dissociation of A and B subunits by 8 M urea on Ultrogel column, followed by dialysis to remove urea). Ni^{2+} also appears to bind to unoccupied Fe and Zn sites since CDPP with lower Fe and Zn contents can be activated by Ni^{2+} to greater extents.

(5) Partial purification and characterization of a Ca^{2+}-inhibited phosphoprotein phosphatase (CIP). The discovery of brain CIP has been reported (Huang, Annual Report, 1983-84). The enzyme has been enriched more than 2000-fold by a procedure involving Affigel Blue, CaM-Sepharose, $(NH_4)_2SO_4$ cut, DEAE, and Sephadex G-100 steps. Further attempts to purify this enzyme beyond the G-100 step resulted in total loss of enzymatic activity. Some known properties of the

partially purified CIP are as follows:

(a) It requires Mg^{2+} for activity, the K_a being ~ 3 mM. With PNPP as substrate, a K_i of 100 μM for Ca^{2+} (in the presence of 3 mM Mg^{2+}) was obtained. The pH optimum is 6.3.

(b) CIP is a rather specific protein phosphatase. Of 10 phosphoproteins tested, 6 did not serve as good substrates: phosphorylase a, phosphorylase kinase, denatured phosphotyrosyl glutamine synthetase, casein, glycogen synthetase, and microtubule associated proteins (MAPS). The 4 phosphoproteins that serve as substrates are: histone H1, histone 2B, synapsin I, and neurofilaments found in the microtubule system. Since histones are artificial substrates and synapsin I and neurofilaments are abundant in the brain, CIP may play an important role in brain. The most interesting observation is the selective dephosphorylation of the two phosphates on synapsin I. The synapsin I used was phosphorylated either at site I by cAMP-dependent protein kinase or at site II by Ca^{2+}-CaM-dependent protein kinase II. Site II phosphate was dephosphorylated by CIP at a low rate which is unaffected by the presence of Ca^{2+}. In contrast, site I was dephosphorylated several times faster by CIP and the dephosphorylation was inhibited by Ca^{2+}. The fact that synapsin I is a poor substrate for CDPP (King et al., J. Biol. Chem. 259, 8080-8083, 1984) seems to indicate that it is not a natural substrate for the CaM-dependent phosphatase. The selective dephosphorylation of site II by CIP suggests that the presence or absence of Ca^{2+} determines when the CaM-dependent kinase and CIP should be activated. CDPP and CIP may function in a coordinated manner in response to the in vivo level of Ca^{2+} ions, thereby forming a subtle regulating system.

Proposed Course:

1. Mechanism of activation of CDPP by Ca^{2+} and other divalent metal ions; search for potential metal carrier protein.

2. Further purification and characterization of the Ca^{2+}-inhibited phosphatase.

3. Ca^{2+} dependence of CaM-phosphodiesterase complex formation.

Publications:

King, M. M., and Huang, C. Y.: Activation of calcineurin by nickel ions. Biochem. Biophys. Res. Commun. 114: 955-961, 1983.

Vita, A., Huang, C. Y., and Magni, G.: Uridine phosphorylase from Escherichia coli B. Kinetic studies on the mechanism of catalysis. Arch. Biochem. Biophys. 226: 687-692, 1983.

Jurgensen, S., Shacter, E., Huang, C. Y., Chock, P. B., Yang, S.-D., Vandenheede, J. R., and Merlevede, W.: On the mechanism of activation of the ATP•Mg(II)-dependent phosphoprotein phosphatase by kinase F_A. J. Biol. Chem. 259: 5864-5870, 1984.

Chock, S. P., and Huang, C. Y.: An optimized continuous assay for cAMP phosphodiesterase and calmodulin. Anal. Biochem. 138: 34-43,, 1984.

King, M. M., Huang, C. Y., Chock, P. B., Nairn, A. C., Hemmings, H. C., Jr., Chan, K.-F. J., Greengard, P.: Mammalian brain phosphoproteins as substrates for calcineurin. J. Biol. Chem. 259: 8080-8083, 1984.

King, M. M., and Huang, C. Y.: The calmodulin-dependent activation and deactivation of the phosphoprotein phosphatase, calcineurin, and the effect of nucleotides, pyrophosphate, and divalent ions. Identification of calcineurin as a Zn and Fe metalloenzyme. J. Biol. Chem. 259: 8847-8856, 1984.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00225-07 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mixed-Function Oxidation of Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Rodney L. Levine Senior Investigator LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Many important enzymes in prokaryotes and eukaryotes are subject to covalent modification mediated by mixed-function oxidation systems. The modified enzymes are frequently rendered catalytically inactive. In the case of bacterial glutamine synthetase, the covalent modification "marks" the protein for subsequent proteolytic degradation by a protease which recognizes the modified protein. A peptide from the modified protein has lost a single histidine residue. Its sequence (MET-HIS*-CYS-HIS-MET) shows it to be rich in chelating residues. This peptide could bind the metal cation required for mixed-function oxidation, thus explaining the site-specific free radical reaction. However, the modified histidine is not the only residue altered upon oxidation. A carbonyl moiety is introduced into a residue located in a different peptide. This carbonyl moiety has been detected in several modified enzymes whose histidine content was unchanged.

The oxidative modification functions physiologically to rapidly inactivate the glycerol dehydrogenase of Klebsiella aerogenes upon shift from an anaerobic to aerobic environment. This inactivation may prevent the accumulation of toxic products which could be produced by the enzyme in an aerobic environment.

The covalent modification produced by mixed-function oxidation was also detected in phosphoglycerate kinase purified from aged rats. Mixed-function oxidation may explain the altered properties noted in many enzymes during aging.

Project Description:

Objectives: Previous years' reports described a covalent modification of glutamine synthetase which is mediated by various mixed-function oxidation systems. Many enzymes and structural proteins now appear susceptible to such oxidations. The physiologic functions remain to be determined, but the modification does appear to "mark" the protein for subsequent proteolytic degradation. The objectives of this project are determination of the chemical nature of the modification; purification and characterization of the enzymes which catalyze the modification and suhsequent proteolysis; and assessment of the physiologic controls which regulate the modification and proteolysis of specific proteins.

Major Findings:

1. Sequence of the peptide containing the modified histidine. We found earlier that the only detectable amino acid alteration caused by the oxidative modification was loss of a single histidine residue. (There are 10 histidine residues in each subunit of glutamine synthetase.) We have now isolated the peptide. Its sequence within the protein is:

MET-HIS*-CYS-HIS-MET

Only the first histidine is modified. The combined chelating effects of these residues presumably directs the "site-specific" free-radical oxidation, by binding the required Fe^{2+}.

2. The modification occurs in aging. Other laboratories have demonstrated changes in key enzymes which occur during aging. These typically include decreased specific activity and altered heat stability. Considerable evidence suggested that the altered enzymes differed only in their conformations; no covalent modifications had been found. Using purified phosphoglycerate kinase from young and old rats (kindly provided by Dr. M. Rothstein), we discovered that "old" enzyme is actually covalently modified. The modification appears similar to a modification which occurs in glutamine synthetase upon mixed-function oxidation. This was demonstrated by showing that both oxidized glutamine synthetase and "old" phosphoglycerate kinase incorporate label from tritiated borohydride, presumahly into a carbonyl moiety. The labeled amino acid products are similar or identical in the two proteins.

3. The covalent modification occurs in vivo. Several investigators in our laboratory demonstrated the susceptibility of many enzymes to mixed-function oxidation in vitro. In collaboration with Dr. E. Lin, we have now shown that the modification occurs in vivo. It had been known for some 25 years that glycerol dehydrogenase of K. aerogenes was rapidly inactivated on transfer from an anaerobic to aerobic environment. The enzyme is inactivated, but cross-reacting material remains. Glycerol metabolism than proceeds via the glycerol kinase pathway, which is induced on exposure to oxygen. We have now demonstrated that active glycerol dehydrogenase does not carry the oxidative modification while the inactivated dehydrogenase is modified. The modification again appears quite similar to that observed for E. coli glutamine synthetase.

4. There is more than one oxidative modification. Thus far, the only detectable change in the amino acid composition of the modified glutamine synthetase was a loss of one of 16 histidine residues. In addition, we had documented that oxidative modification leads to the introduction of a carbonyl moiety. Both of these changes paralleled the loss of catalytic activity of the enzyme. While this made it likely that the carbonyl moiety was on the modified histidine, it is actually on a different residue. This was demonstrated by labeling the carbonyl with borotritide or with 2,4-dinitrophenylhydrazine. After cleavage with trypsin or CNBr, the peptide containing the derivatized carbonyl was distinct from the peptide bearing the modified.

Studies by Dr. E. Stadtman demonstrate that many polyamino acids are susceptible to mixed-function oxidation. Further, certain enzymes which are oxidatively inactivated do not demonstrate any change in histidine content. They are covalently modified with the generation of carbonyl moieties (glycerol dehydrogenase, lysozyme, phosphoglycerate kinase, and ribonuclease). The altered residues have not been identified with certainty, but lysine appears a likely candidate.

5. Development of rapid assays. The study of this covalent modification should be facilitated by simple, sensitive analytical methods. A technique was developed for labeling of the carbonyl with tritiated borohydride, based on published procedures. While not specific for carbonyl on amino acids, the method has proven simple and sensitive. It also provided a label which could be followed during studies on peptide fragments. Similarly, we have adapted procedures for the precolumn derivatization of amino acids for use in reverse-phase HPLC systems. Amino acid analyses now require only 13 minutes. Coupling the two methods, one can label a protein with tritium, perform rapid hydrolysis, and then amino acid analyses -- within one day.

Significance to Biomedical Research and the Program of the Institute:

Studies in this and other laboratories provide a growing list of proteins which are susceptible to oxidative modification. We have implicated this modification as a "marker" for intracellular proteolytic turnover. While this is an important physiologic role, there may be other physiologic functions mediated by this covalent modification. These include host defense mechanisms such as those provided by the oxidative burst of neutrophils; oxygen toxicity, both endogenous and iatrogenic, such as bronchopulmonary dysplasia and retrolental fibroplasia; and in the aging process, in which it appears that a number of enzymes accumulate in an inactive form, perhaps due to an oxidative modification. This may be a particularly interesting discovery because evidence to date has ruled against the occurrence of posttranslational covalent modification in the "aged" proteins. Such modifications certainly occur in the case of "old" rat phosphoglycerate kinase

Proposed Course:

Characterization of the chemical nature of the modified histidine remains the immediate, key objective. With isolation of the purified peptide containing the modified residue, it should be possible to determine the structure using techniques such as NMR and mass spectroscopy. However, the discovery of at least one other modified residue in glutamine synthetase (and other proteins) requires

characterization of that residue. Most importantly, the involvement of this modification in aging and in physiologic regulation will continue to be studied.

Publications:

Levine, R. L: Mixed-function oxidation of histidine residues. Meth. Enzymol. 107: in press, 1984.

Levine, R. L. and Lehrman, S. R.: Identification of amino acid phenylthiohydantoins by multicomponent analysis of ultraviolet spectra. J. Chromatog. 288: 111-116, 1984.

Levine, R. L.: Oxidative modification of glutamine synthetase: I. Inactivation is due to loss of one histidine residue. J. Biol. Chem. 258: 11823-11827, 1983.

Levine, R. L.: Oxidative modification of glutamine synthetase: II. Characterization of the ascorbate model system. J. Biol. Chem. 258: 11828-11833, 1983.

Martensen, T. M., Levine, R. L., and Sliwkowski, M. X.: Tyrosine phosphate and tyrosine sulfate in proteins. In Chaiken, I. M., Wilchek, M., Parikh, I. (Eds.): Affinity Chromatography and Biological Recognition. New York, Academic Press, 1983, pp. 401-403.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00237-05 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Toxicity and Transport of Bilirubin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Rodney L. Levine Senior Investigator LB, NHLBI

COOPERATING UNITS (if any)

Laboratory of Neurosciences
National Institute on Aging

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

(a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hyperbilirubinemia is probably the most frequently diagnosed and treated condition in the human newborn. Treatment is aimed at preventing the entry of bilirubin into the brain, because of the risk of permanent neurologic damage. The mode by which bilirubin enters the brain, its metabolic fate after entry, and the sites of toxic action are unknown. Using an osmotic stress, we opened the blood-brain barrier in rats, permitting the entry of albumin-bound bilirubin, and thereby creating an animal model of human kernicterus. We performed a pharmacokinetic study of the clearance of bilirubin from the brain. Contrary to expectations from human clinical observations, the clearance of bilirubin was very rapid, with a half-time of 1.6 hours. This half-time was the same as that for clearance from the serum, suggesting that brain bilirubin was removed by transport or diffusion back into the general circulation. Thus, in the undamaged rat brain, bilirubin is rapidly cleared. The potential for clearance of bilirubin from human neonatal brain should be considered.

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Project Description:

Objectives: Bilirubin is an end-product of heme metabolism in humans. In vitro, bilirubin is toxic to cells and to isolated mitochrondria. The biochemical basis of the toxicity is unknown. In vivo, bilirubin is remarkably benign, with the notable exception of newborns. While most infants suffer no lasting sequelae, a few develop bilirubin encephalopathy, or kernicterus. The mode of entry of bilirubin into the brain and the site of toxic action are unknown. The objective of this study is to understand these mechanisms of transport and toxicity.

Major Findings:

We previously demonstrated that osmotic opening of the blood-brain barrier permits entry of albumin-bound bilirubin into the brain of rats, providing a potential experimental model for kernicterus. We have now completed studies of the time course of entry and removal of bilirubin, following the osmotic treatment. The purpose of these studies was to define the optimum time for our planned experiments on the metabolic effects of bilirubin on cerebral metabolism.

Based primarily on human, neonatal autopsy results, current thinking holds that once bilirubin enters the brain it precipitates and can be removed only very slowly. We had thus expected to find the bilirubin content in the rat brains rising to a plateau, and then holding rather steady. The results were quite different. Based on studies in 50 animals, it is clear that the bilirubin is removed very rapidly from the rat brain. Further, clearance from the brain parallels that from the general circulation. Both have a half-time of 1.6 hours. Thus, the most likely mechanism for removal of cerebral bilirubin is transport or diffusion back into the general circulation.

These studies required the development of sensitive, selective, and convenient assays for bilirubin. They were based on multicomponent analysis of visible range spectra. Preliminary studies show that the assays are also useful in the clinical laboratory, particularly for the determination of amniotic fluid bilirubin.

Significance to Biomedical Research and the Program of the Institute:

Current treatment of neonatal hyperbilirubinemia is generally based on untested assumptions: (1) bilirubin enters the brain in its unbound form; (2) once in the brain, removal of bilirubin is very slow or nonexistent. Both these assumptions may be invalid. The second one is incorrect, if the metabolic capability noted in the rat also exists in human neonates. In the undamaged rat brain, bilirubin is rapidly cleared, in contrast to its persistence in autopsy proven human kernicterus.

The potential for clearance of bilirubin from human neonatal brain should be investigated, especially in the absence of underlying tissue damage.

Proposed Course:

Having established the kinetics of entry and exit of bilirubin, we can now utilize the rat model to probe the metabolic effects of bilirubin on cerebral metabolism using the 2-deoxyglucose technique. The loci and mechanism of bilirubin toxicity remain the focus of this research.

Publications:

Levine, R. L.: Bilirubin and the blood-brain barrier. In Levine, R. L. and Maisels, M. J. (Eds.): Hyperbilirubinemia in the Newborn, Report of the Eighty-fifth Ross Conference on Pediatric Research. Columbus, Ohio, Ross Laboratories, 1983, pp. 125-129.

Levine, R. L.: The toxicology of bilirubin. In Levine, R. L. and Maisels, M. J. (Eds.): Hyperbilirubinemia in the Newborn, Report of the Eighty-fifth Ross Conference on Pediatric Research. Columbus, Ohio, Ross Laboratories, 1983, pp. 39-44.

Levine, R. L. and Maisels, M. J. (Eds.): Hyperbilirubinemia in the Newborn, Report of the Eighty-fifth Ross Conference on Pediatric Research. Columbus, Ohio, Ross Laboratories, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00239-05 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Regulation of Glutamine Synthetase in E. coli and S. cerevisiae

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Sue Goo Rhee	Research Chemist	LB, NHLBI
	Ja Hyun Koo	Visiting Fellow	LB, NHLBI
	Sang Chul Park	Visiting Fellow	LB, NHLBI
Others:	Kang-Wha Kim	Visiting Fellow	LB, NHLBI
	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.3	3.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

(1) The glnD gene which encodes uridylyltransferase-uridylyl-removing enzyme (UT-UR) in E. coli was cloned into a plasmid vector carrying the strong regulatable λ phage promoter pL such that UR-UR was overproduced to the levels approaching 800-fold. Induction of this enzyme was optimized by varying the induction temperature and the growth medium. Detailed restriction maps and the transcriptional direction of the glnD gene were also established.

(2) The promoter region of the glnD gene was inserted into a promoter expression vector and its transcriptional control was studied by measuring galactokinase transcribed from this promoter. The result indicates that the glnD gene is metabolically regulated.

(3) The glnE gene (structural gene for adenylyltransferase in E. coli) was subcloned into pBR322 and subsequently into the pL vector. Nearly 300- to 400-fold amplification in the synthesis of adenylyltransferase was achieved.

(4) Crude extracts of Saccharomyces cerevisiae were separated into two peaks containing glutamine synthetase with different catalytic properties. Glutamine synthetase in the earlier peak exhibited a higher ratio of biosynthetic to (nonphysiological) transferase activity than that in the latter peak, and the ratio difference became more pronounced upon aging. The glutamine synthetase of higher biosynthetic activity was purified to apparent homogeneity.

PHS 6040 (Rev. 1/83)

GPO 911-917

Project Description:

Objectives:

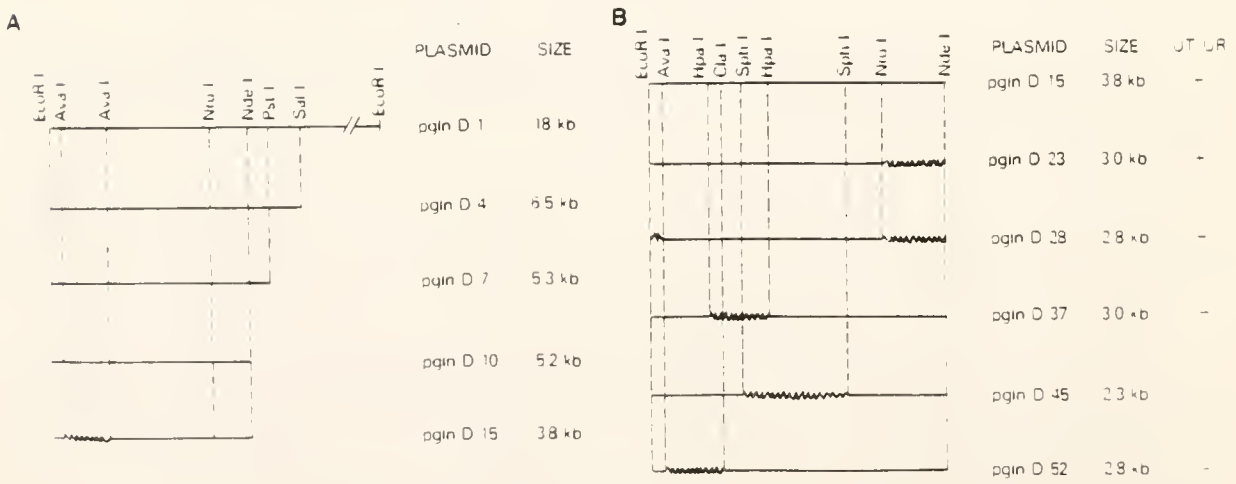
(1) To study the regulation of two bifunctional enzymes, adenylyltransferase and uridylyltransferase-uridylyl-removing enzyme. Our immediate goal is to obtain strains which can overproduce these enzymes.

(2) To understand how yeast rapidly inactivates glutamine synthetase upon exposure to a nitrogen excess medium.

Major Findings:

(1) The converter enzyme, UT-UR, catalyzes the interconversion of P_{II} and P_{II}-UMP. This bifunctional enzyme senses the fluctuation in the availability of nitrogen source and accordingly affects the activity of glutamine synthetase through the regulatory protein P_{II}. In addition, genetic evidence indicates that the metabolic information apprehended by the coupled system of P_{II} and UR-UR can be integrated to the complex system involved in the regulation of the synthesis of glutamine synthetase.

In order to make it possible to study this important enzyme which exists in extremely low concentration, the glnD gene (structural gene for UT-UR) was cloned into a plasmid vector carrying the strong, thermoinducible λ phage promoter pL. A small DNA fragment containig the glnD gene was obtained through a series of subcloning, as summarized in scheme 1A. Further localization of the glnD gene on pglnD15 was achieved by several deletion experiments as depicted in scheme 1B. Combining these results, it was concluded that the AvaI-NruI fragment (2.5 kb) comprises the glnD gene the size of which is estimated to be 2.0 kb based on the molecular weight of UT-UR, 95,000 daltons.



The pBR322 derivatives generated during the process of reducing the size of the fragments containing the glnD gene from the 18 kb of pglnD1 to 2.3 kb of pglnD28 differ from each other not only in the size of the insert but also in the insertion position. However, the UR activity in the strains harboring these plasmids was similar, i.e., 10- to 20-fold of the wild type E. coli K-12, suggesting that the glnD gene was expressed mainly under the control of its own promoter. The strength of the glnD gene promoter seems to be weak: despite the elevated level of UT-UR, this protein was still a minor component in the crude extract prepared from a strain bearing pglnD15 such that protein band corresponding to the molecular weight of UT-UR was not recognizable at all in an SDS-polyacrylamide gel; the radiolabeled band of UT-UR was extremely weak compared to that of penicillinase encoded by the ampicillin resistance gene in the same pglnD15 when the plasmid encoded gene products were specifically labeled using [^{35}S]methionine in the "maxicell" system. To further elevate the synthesis of UT-UR, the glnD gene was cloned into the plasmid vector pKC30 which carries the strong pL promoter. Control over the activity of the pL is achieved by using host strains that supply a thermolabile repressor from the λ cI857 gene present on the chromosome. To place the glnD gene under the control of pL promoter, the EcoRI-NdeI fragment (3.8 kb) of pglnD15 was treated with Bal31 exonuclease for a short period of time, filled in with Klenow enzyme, and then blunt-end ligated to the HpaI site of pKC30. The resultant pglnD110 was transformed into four different hosts: two N^+ strains, M5219 and N4830; N^- strain, N4831; and cI^- strain, RB9081. The UR activities measured in these transformants are summarized in Table I. These results indicate that the repressor protein (cI) and antitermination protein (N) are necessary for maximal expression of the glnD gene.

Table I.
Overproduction of UT-UR by Various Host Cells Bearing pglnD110

Host strain	UR overproduction ratio
M5219	300-800
N4830	300-400
N4831	150-250
RB9081	100-200

Cells were initially grown in LB medium at 30°C and at A_{600} = 0.5, the temperature was raised to 42°C for 5 ~ 6 hours. The ratios of UR overproduction are relative to the enzymic activity in wild type E. coli K-12. Relatively wide variations in the ratios are mainly due to the difficulty in measuring accurately the UR activity of wild type E. coli K-12.

During the studies on time-dependent induction of UR, it was learned that UR synthesized in cells grown on LB is unstable over the time period of 12 hours at 42°C, while it remained active in cells grown on minimal medium. At the present time, we do not know what causes this uneven decay of UR activity.

(2) When a strain (YMC10) bearing pglnD15 was grown on several different mediums, the UR activity varied (Table II): its level was highest with a minimal medium containing limited nitrogen; decreased by 40% in response to the addition of excess ammonia; and reduced to one-ninth when grown on LB medium. We examined

the possible transcriptional regulation of the glnD gene by utilizing the gene fusion techniques. Hybrid plasmid pHB74 was constructed by localizing the promoter region of the glnD gene upstream of the galactose kinase (galK) gene. The activity of galactose kinase measured in a plasmid-bearing strain (N100) varied in parallel to the UR activity in response to the growth medium (Table II). This result suggests that the transcription of the glnD gene is metabolically regulated. However, the nature of the control signals and the significance of the control are not known yet.

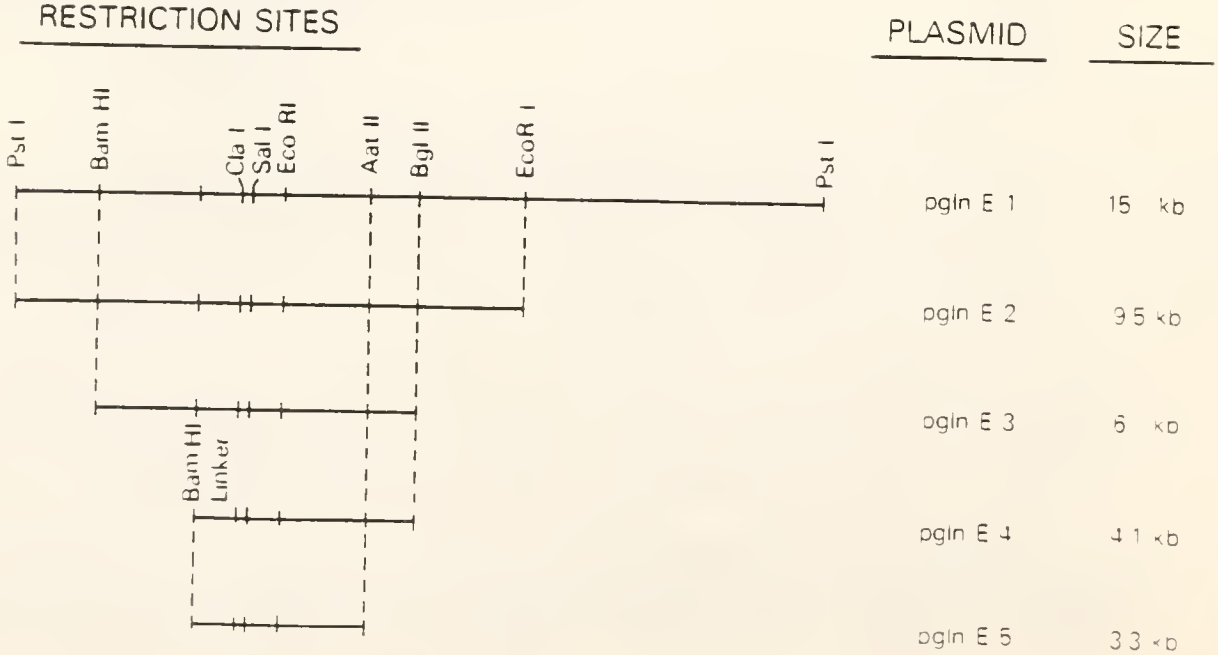
Table II

Effect of Growth Medium on the glnD Promoter Function

		Relative enzyme activity		
Enzyme measured	Host cell/plasmid	Nitrogen limiting	Nitrogen excess	LB
UR	YMC10/pglnD15	1	0.6	0.11
Galactokinase	N100/pHB74	1	0.7	0.25

Cells were grown at 37°C and at A_{600} = 0.5, harvested for the enzymic assays. The UR activities were corrected for the protein concentration and the galactokinase activities for the cell density. The corrected activities of both UR and galactokinase were normalized by taking the activities from the nitrogen limiting minimal medium as 1.0. The nitrogen limiting minimal medium contained 4 mM glutamine and 0.5% glucose; the nitrogen excess minimal medium contained 20 mM ammonium chloride, 4 mM glutamine and 0.5% glucose; the LB is the Luria broth medium.

(3) The cascade regulation of glutamine synthetase in E. coli involves another bifunctional enzyme, adenylyltransferase. The structural gene (glnE) for this converter enzyme was also subcloned (scheme 3), and cloned into the pL vector



for the purpose of overproduction. In this way, a strain which produces as much as 300- ~ 400-fold of the enzyme was obtained.

(4) Glutamine synthetase in yeast responds to excess glutamine through both repression and inactivation. It has been known that inactivation results in a loss of biosynthetic activity but not of (nonphysiological) transferase activity. However, the inactivation mechanism is not yet known. In an effort to understand the nature of the inactivated enzyme, we looked for a method to separate active and inactive forms. Subsequently, it was found that chromatography of crude extracts on DEAE (either conventional or HPLC) columns yields two separated GS peaks with different catalytic properties. The ratio of biosynthetic to transferase activity is 0.12 for the first GS peak and ~ 0.08 for the second GS peak when assayed just after the elution; but upon incubation, the second peak becomes broader and the activity ratio decreases continuously until no more biosynthetic activity can be found in the second peak, while the ratio remained unchanged with the first peak. Although highly probable, it is not clear yet whether GS in the second peak is also involved in in vivo inactivation. Glutamine synthetase from the first peak was purified to homogeneity. Two column steps, DEAE-cellulose and hydroxylapatite column chromatography, were used.

Proposed Course:

(1) Adenylyltransferase and uridylyltransferase will be purified from the overproducing strains. The interaction with various ligands and proteins such as P_{II} and glutamine synthetase will be studied.

(2) A mechanism to inactivate glutamine synthetase in yeast in response to excess glutamine synthetase will be further pursued.

Publications:

Suh, S. W., and Rhee, S. G.: Preliminary X-ray crystallographic studies and molecular symmetry of the P_{II} regulatory protein from E. coli. J. Biol. Chem. 258: 10294-10295, 1983.

Rhee, S. G.: 5'-Nucleotidyl-O-tyrosine bond in glutamine synthetase. Meth. Enzymol. 107: 183-200, 1984.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: Nucleotidylation involved in the regulation of glutamine synthetase in E. coli. In Freedman, R. (Ed.): Enzymology of Posttranslational Modification of Proteins. New York, Academic Press, Vol. II, 1984, in press.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: E. coli glutamine synthetase. Meth. Enzymol., 1984, in press.

Chung, H. K., and Rhee, S. G.: Separation of glutamine synthetase species with different states of adenylylation by chromatography on monoclonal anti-AMP antibody affinity columns. Proc. Nat. Acad. Sci. U.S.A, 1984, in press.

Chung, H. K., Park, S. C., and Rhee, S. G.: Monoclonal antibodies specific to E. coli glutamine synthetase. J. Biol. Chem., 1984, in press.

Rhee, S. G., and Park, S. C.: Uridylyltransferase-uridylyl-removing enzyme in the regulation of glutamine synthetase in E. coli. Curr. Top. Cell. Regul., 1984, in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 HL 00240-05 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Purification and Characterization of Selenium-containing Transfer RNAs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Wei-Mei Ching, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS (if any)

C. S. Chen, (Institute of Botany, Academia Sinica, Taiwan)
Ronald Peterson, (Uniformed Services University of the Health Sciences)

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to study the codon- anticodon interaction of seleno-tRNA^{Glu} from Clostridium sticklandii, glutamate codons GAA and GAG were synthesized. Under near physiological condition, seleno-tRNA^{Glu} interacts almost equally well with both codons, while Escherichia coli tRNA$_2^{Glu}$, which contains 5-mnm2-s-U instead of 5-mnm-2-se-U at the wobble position, showed a profound preference of GAA over GAG. Similar results were observed at lower pH (pH 5.5). Deselenization of seleno-tRNA^{Glu} abolished the codon stimulated ribosome binding activity. In a cell free protein translation system, [^{3}H] Glu-seleno-tRNA^{Glu} is a very active substrate for the incorporation of glutamate into the nascent protein. Genomic DNA fragments of C. sticklandii (approximately 4 K bp), which contained the seleno-tRNA^{Glu} gene were ligated to plasmid puc 8 and used to transform E. coli cells (strain HB 101). Two positive clones have been found. This serves as an effective starting point for further work at the genetic level. Expression of the cloned seleno-tRNA^{Glu} gene may produce significant amount of tRNA precursors which can be modified with selenium.

The presence of selenium-containing tRNAs in various rat tissues (heart, kidney, testis and liver) was investigated. Bulk tRNAs prepared from liver contained the highest amount of selenium (~ 0.6%). Many different selenium-containing species which can be resolved on a HPLC DEAE column were present in the preparations. Although the selenium component(s) are alkaline labile, they are not seleno amino acids because no selenomethione or selenocysteine was detected.

The amount of seleno-tRNAs in the total tRNA populations of a cultured plant cell line, Daucus carota, ranged from 0.54% to 1.2%. Total nucleoside analysis of bulk tRNAs indicated that the selenium-containing component(s) were very hydrophobic in character.

GPO 904-917

Progress:

I. Biological function of seleno-tRNA^{Glu}

(i) codon recognition: previous results indicate that at high, nonphysiological concentration of magnesium, (20mM), seleno-tRNA^{Glu} from C. sticklandii recognizes both glutamate codons (GAA and GAG) equally well, and does not respond to the termination codons, UAA and UAG. In order to further investigate the codon-anticodon interaction at a near physiological condition, trinucleotides GAA and GAG were synthesized by the method of Thach with some modifications: GpA was mixed with ADP(GDP) in the presence of polynucleotide phosphorylase. After incubation at 34° C for 24 hours trinucleotide UAA (UAG) were separated from the reaction mixture by HPLC reversed phase chromatography.

(a) At near physiological concentration of magnesium (10mM) seleno-tRNA^{Glu} interacts with both codons with a slight preference for GAA, the difference is small but is reproducible. This is true at either pH 7.3 or pH 5.5. Nevertheless, E. coli tRNA$_2^{Glu}$, which contains 5-mnm-2-s-U, instead of 5-mnm-2-Se-U showed a 4-fold preference for GAA at pH 7.3 and 2-fold at pH 5.5 under the same experimental conditions.

(b) When seleno-tRNA^{Glu} was deselenized by CNBr treatment, no response to glutamate codons was observed. Similar results have been reported for E. Coli tRNA$_2^{Glu}$.

(ii) Cell free protein translation: Wheat germ cell extract (using mouse globin message as mRNA) was used to test the activity of [^{3}H] Glu-seleno-tRNA^{Glu} from C. sticklandii as a donor of glutamate for protein synthesis. Over 40% of the added [^{3}H] was incorporated into protein within 60 min, while essentially no incorporation was observed in the absence of mRNA.

II. Cloning of seleno-tRNA^{Glu} gene

In several instances it has been shown that the primary transcript of a tRNA gene is not a mature tRNA and some nucleoside modifications occur in a strict order which precisely correlates with the size alterations of the tRNA precursors. In order to study the pathway of seleno-tRNA^{Glu} maturation, especially the modification with selenium, cloning of the seleno-tRNA^{Glu} gene from C. sticklandii was undertaken (collaboration with Dr. Ronald Peterson at USUHS).

Digestion of genomic DNA with restriction enzyme Hind III generated 4 K bp fragments which would hybridize with (^{32}P)-3'-end-labeled seleno-tRNA^{Glu}. These 4 K bp fragments were ligated to plasmid puc 8 and used to transform E. coli (strain HB 101). Two positive clones have been found. Digestion of the cloned DNA and subcloning of a shorter fragment ($\leq$ 400 bp) is in progress.

III. Seleno-tRNAs in animal tissues:

Information from animal studies indicates that depletion of selenium is a slow process. In order to obtain a high incorporation of ^{75}Se into tRNAs, Sprague-Dawley rats were kept on selenium-deficient diets (<0.01 µg/g) for

5 weeks before the injection of $H_2{}^{75}SeO_3$. Various tissues were examined for the presence of selenium-containing tRNAs.

(i) Bulk tRNAs prepared from liver contained the highest amount of selenium (~ 0.6%). On a HPLC ion exchange (DEAE) column, many different selenium-containing species were resolved. This pattern is very different from the pattern observed for cultured mouse leukemia cells.

(ii) The selenium component(s) in the rat liver tRNAs is alkali labile but more stable than the components found in cultured mouse leukemia cells. A substantial amount of selenium (32%) remained with rat liver tRNA after 1 hour incubation at pH 8.5 (0.5M Tris. HCl). It is unlikely that the selenium components are seleno amino aids attached to the tRNA via acyl bond because (a) when bulk tRNA was deacylated and analyzed on an amino acid analyzer, no significant ^{75}Se eluted with the carrier selenomethione; (b) when bulk tRNA was deacyled and carboxymethylated, again only an insignificant amount of ^{75}Se eluted with carboxymethylselenocysteine whereas the recovery of added carrier selenocysteine was 45-65% following carboxymethylation.

IV. Selenium-containing tRNAs from Daucus carota L.

Samples of carrot tRNA obtained from Dr. C. S. Chen, (Institute of Botany, Academia Sinica, Taiwan) were very unstable. The amount of seleno-tRNAs in the total tRNA populations ranged from 0.54% to 1.2% as determined by atomic absorption. Total nucleoside analysis on a reversed-phase HPLC column indicated that the selenium containing component(s) interacts very strongly with the column matrix.

Proposed action:

(I) After sub-cloning of the seleno-$tRNA^{Glu}$ gene, its DNA sequence will be determined and the total tRNA sequence can be deduced from this information. Whether a tRNA gene from anaerobic bacteria contains intervening sequences and/or is clustered with other tRNA genes will also be answered. Expression of this cloned gene may produce significant amounts of precursor(s) which can be modified with selenium.

(II) The nearly equal recognition of glutamate codons GAA and GAG by seleno-$tRNA^{Glu}$ from C. sticklandii appears surprising. It can not be explained by a weak H-bonding nor by the simple ionization of the seleno group. Since thio-$tRNA^{Glu}$ and seleno-$tRNA^{Glu}$, thio-$tRNA^{Cys}$ and seleno-$tRNA^{Lys}$ from E. coli (prepared by A. Wittwer) seem to differ by only one modification (thio vs. seleno their response to synonymous codons will be investigated under identical conditions. These results may elucidate the special effect of Se on codon-anticodon interaction.

(III) A computer search for E. coli proteins rich in glutamate or lysine or both near the N-terminal is in process. The synthesis of these proteins will be studied in a cell free translation system which could be tRNA dependent. The effects on translation efficiency using either thio or seleno containing glutamate (lysine) accepting tRNAs will be compared. This information, together with those obtained in (II) will contribute to the better understanding of the biological functions of Se.

(IV) Previous reports suggested that both C. sticklandii and D. Carota have a seleno-tRNA species which cochromatographs with proline accepting activity. Whether there is a seleno-tRNA^{Pro} may be answered by employing the anti-AMP antibody column. Bulk tRNAs pre-enriched in Se will be charged with proline and fractionated on the antibody column. The relative amounts of Se, A_{260}, and proline accepting activity in the void volumn fraction can clarify this question.

Honors:
Received a Chinese-American Fellowship in preventive Medicine (awarded by the American Health Foundation) to attend the Third International Symposium of Se in biology and Medicine which was held in Beijing, China, May 27-June 1 1984.

Publications:

Ching, W.M., Wittwer, A.J., Tsai, L., and Stadtman, T.C.: The distribution of two seleno-nucleosides among the selenium-containing tRNAs from Methanococcus vannielii. Proc. Natl. Acad. Sci. U.S.A. 81, 57-60 (1984).

Ching, W.M.: The occurrence of selenium-containing tRNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 81, 3010-3013 (1984).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00241-05 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Detection and Regulation of Phosphotyrosine Modification in Cellular Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Todd M. Martensen Guest Worker LB, NHLBI

COOPERATING UNITS (if any)

Laboratory of Vision Research, NEI
Laboratory of Cellular Metabolism, NHLBI

LAB BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1	0.8	0.2

CHECK APPROPRIATE BOX(ES)

(a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The phosphorylation of protein tyrosine residues is closely linked to cell growth and transformation. Methodological obstacles have been overcome which allow the measurement of protein tyrosine kinase and protein phosphotyrosine phosphatase in cells. The assay has been used to quantify protein tyrosine kinase activity in normal and malignant cells. Protein tyrosine kinase activity was found to be associated with increased cell division, and/or activated in the transformed state but not in the normal state by particular growth factors. These features may be important in regulating protein tyrosine phosphorylation in vivo. Assay of protein phosphotyrosine phosphatase activity (PTPase) has been carried out in normal and cancerous cells using radioactive phosphotyrosine glutamine synthetase. Characteristics of the PTPase activity in Ehrlich Ascities Tumor (EAT) cells are: the activity is predominately cytosolic and dilution-dependent with maximal activity ~ 5 nmol/min/mg. Heat stable nondialyzable inhibitors of the PTPase were found and characterized in boiled cell extracts which could act as regulatory molecules. The substrate was used to characterize the protein phosphotyrosine phosphatase activity of calcineurin: Antibodies which recognize tyrosine phosphate have been successfully used to develop immunological detection methods for phosphotyrosine containing proteins.

Project Description:

Objectives: The role of proteins containing Tyr-P in eucaryotic cellular function is of considerable interest since it was found that a retrovirus gene transforming product was a protein tyrosine kinase. Also, receptors for insulin and several growth receptors (epidermal, platelet, and two tumor types) display protein tyrosine kinase activity, presumably as an intrinsic part of their receptor. Four primary goals were set to allow biochemical investigation of the interconverting enzymes and their substrates:

1. Detection and quantification of Tyr-P in proteins.

2. Assay of the enzymes responsible for the phosphorylation of protein tyrosine residues.

3. Assay of the enzymes responsible for the dephosphorylation of protein Tyr-P residues.

4. Production of antibodies which recognize Tyr-P residues in proteins.

Major Findings:

The stability of the phosphoryl bond of Tyr-P whether free or in peptide linkage to base digestion was shown to allow 80% recovery. This allowed an assay to be developed for protein tyrosine kinase which was extremely sensitive and could be used to measure this activity in cultured cells or biopsy samples. Measurement in normal and transformed cells was done to determine the role of the kinase in certain disease states since regulation of the kinase activity may be fundamental to cell growth and transformation. Measurement of PTK activities in retina and malignant retinoblastoma showed the both activites were comparable in cell extracts. However the retinoblastoma PTK activity was stimulated by a growth factor from the cells while the PTK activity was not. The responsiveness of the cancer cell PTK activity to the factor may be involved with the transformed state. Comparison of PTK and PTPase activites in non malignant tissue proliferative disease of skin (psoriasis) was studied to determine their association with cell division. Both activities were found to increase in psoriatic skin samples compared to normal skin from the same individual. The PTK activity of the psoriatic tissue was activated by epidermal growth factor; the same activity in adjacent tissue did not. These studies indicate that PTK activity is associated with cell division. The steady state levels of phosphorylated substrates is controlled by the relative activities of the PTK and PTPase. Regulation of both activities could result in a increased levels of phosphorylated substrates whose identity and function are not well understood.

Opposing the kinase reaction is a unique protein phosphatase (PTPase) which dephosphorylates protein phosphotyrosine residues. This activity is being studied in normal and cancerous cells to determine if the activity or its regulation can be correlated with disease states. The protein phosphotyrosine phosphatase activity is high in all tissues studied, but varies significantly depending on tissue type. In EAT cells, the phosphatase appears to be strongly repressed by heat stable endogenous inhibitors.

In cell extract higher PTPase specific activities were obtained with increasing dilution of extract prior to assay, suggesting the presence of putative inhibitors of the PTPase. The PTPase was not inhibited by heat stable protein phosphatase inhibitors I_1 or I_2. Boiled cell extracts were found to contain nondialyzable material stable to TCA and $(NH_4)_2SO_4$ precipitation which inhibited the PTPase. The material migrated as a 28,000 molecular weight species on size exclusion HPLC. Spectral characterization of inhibitory fractions after HPLC reverse phase chromatography showed nucleic acid to be present. The inhibitory material was insensitive to trypsin but labile to RNase. E. coli-tRNA ($M_r \sim 26 \times 10^3$) was found to inhibit the PTPase ~ 50% when GSTP was 10 μM. The polymeric structure of tRNA appears to be essential for its inhibitory capacity since after nuclease treatment it no longer inhibits. The ability of RNA polymers to regulate protein phosphotyrosine interconversion could have important consequences for cellular growth and differentiation. Calcineurin which displays protein phosphatase activity dephosphorylated [^{32}P]phosphotyrosyl glutamine synthetase. Phosphatase activity with and without calmodulin was almost completely dependent on Mn^{2+}; with Ca^{2+}, even in the presence of calmodulin, activity was very low. In the presence of Mn^{2+} without added Ca^{2+}, calmodulin stimulated activity three-fold; this activation was inhibited by the calmodulin antagonists, trifluoperazine and W-7. Phosphotyrosine was somewhat more potent than phosphoserine or phosphothreonine as an inhibitor of phosphoprotein hydrolysis; at a concentration of 20 mM all three inhibited calmodulin-supported phosphatase activity. Although proportional to enzyme concentration, the phosphatase activity in the presence of calmodulin was not constant with time. After an initial period of 2-3 min, the rate declined markedly. This time course was not observed in the absence of calmodulin, i.e., the basal reaction role was constant. The Michaelis constant for substrate (3 μM) was identical whether measured at 2 or 12 min in assays with calmodulin suggesting that the change in the rate of hydrolysis did not result from a large decrease in affinity for the phospholprotein. The V_{max} was (~ 10 nmol/min/mg) is low for a protein phosphatase.

Antibodies which bind Tyr-P (Anti Tyr-P) were produced in sheep by injection of Tyr-P covalently bound to albumin. These antibodies were affinity purified on columns of agarose containing bound Tyr-P. These antibodies were shown to bind Tyr-P at micromolar concentrations, and protect the dephosphorylation of phosphotyrosyl glutamine synthetase by alkaline phosphatase. Considerably progress has been made in the ability to use the antibody with immunological techniques to detect proteins containing tyrosine phosphate. Electrophoretic transfer of phosphotyrosine glutamine synthetase to nitrocellulose followed by incubation with Anti Tyr-P led to the formation of a stable complex which was detected by anti sheep IgG antibodies convalently bound to peroxidase. This technique of detection has been shown to detect approximately 300 fmol of CMGSTP. This method should allow the detection and isolation of the physiological substrates of phosphotyrosine kinases.

Significance to Biomedical Research and the Program of the Institute:

The control of cell growth and transformation has been related to the phosphorylation of tyrosine residues. Understanding the role and regulation of this activity will be useful to understanding a variety of human diseases at the molecular level.

Proposed Course:

The regulation of the enzymatic activities which interconvert Tyr-P residues in cellular proteins remains to be elucidated. Since the known tyrosine kinases contain a phosphorylated tyrosine residue, it is possible that this phosphorylation site affects enzymatic activity. The lability of the kinase in extracts if due to dephoslphorylation should be prevented by specific phoslphatase inhibitors, alternative substrates for the phosphatase, and antibodies which protect Tyr-P residues from phosphatase attack. The capability to carry out these studies is now available. Secondly, the isolation and characterization of substrates for the kinase by the use of affinity column of immobilized Anti Tyr-P antibodies has yet to be undertaken. The role of tyrosine phosphorylation in cell growth and transformation requires the identification of the cellular activities of the substrates which at this time is basically a matter of guesswork.

Publications:

Martensen, T. M.: Chemical properties, isolation, and analysis of O-phosphates in proteins. Meth. Enzymol. 107: 3-23, 1984.

Gentleman, S., Martensen, T. M., Digiovanna, J. J., and Chader, G. J.: Protein tyrosine kinase and protein phosphotyrosine phosphatase in normal and psoriatic skin. Biochimica et Biophysica Acta, 798: 53-59, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00242-04 LB

PERIOD COVERED

September 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis and Properties of Selenium-containing tRNA from Escherichia coli.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Arthur J. Wittwer, Staff Fellow, Laboratory of Biochemsitry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

When grown in the presence of trace amounts of selenium, Escherichia coli synthesize lysine and glutamate accepting species of tRNA which contain the selenonucleoside, 5-methylaminomethyl-2-selenuridine (mnm5se2U). The selenonucleoside was quantitated in bulk, hydrolyzed tRNA by monitoring at 313 nm during reversed phase high performance liquid chromatography. E. coli tRNA from cells grown in rich, natural media such as beef extract or tryptone-yeast extract, without intentional selenium supplementation, contained 4 to 61 pmol mnm5se2U per A260 unit of tRNA. Thus, there is probably enough selenium present in natural environments for mnm5se2U synthesis to occur. In tRNA from cells grown on selenium-supplemented media (1 μM selenite or selenate) a maximum of 60-70 pmol mnm5se2U per A260 unit was observed, or about 4 mol mnm5se2U per 100 mol tRNA. Under these conditions an approximately equal amount of 5-methylaminomethyl-2-thiouridine (mnm5s2U) was also present. The amount of mnm5se2U did not increase and the amount of mnm5s2U did not decrease from these values when the culture medium selenium was increased (or if sulfur was decreased) to levels which were inhibitory to growth. A mutant defective in the synthesis of mnm5s2U (obtained from M. G. Marinus) incorporated the same amount of selenium into tRNA as the parent strain. The selenonucleoside in the mutant was identified as 5-aminomethyl-2-selenouridine. Studies with wild-type cells, employing chloramphenicol and rifampicin, suggested that a stable tRNA species existed in selenium-deprived E. coli which could be modified with selenium to form mnm5se2U-containing tRNA. By labeling mnm5s2U residues with S-35 it was demonstrated that synthesis of mnm5se2U was accompanied by a loss of sulfur from mnm5s2U residues. Finally, it was shown that purified E. coli tRNA-Lys or tRNA-Glu, containing mnm5s2U, could serve as a substrate in a crude, in vitro, selenium incorporation system. Synthesis of mnm5se2U in this system was completely dependent upon addition of ATP. This is the first observation, in vitro, of a specific enzymatic incorporation of selenium into a macromolecule.

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Project Description:

Previous work has shown that E. coli synthesize ^{75}Se-labled tRNA when grown with trace amounts (0.1 to 1.0 μM) of $^{75}SeO_3^{2-}$ or $^{75}SeO_4^{2-}$. Incorporation of selenium is not due to non-specificity of a modification process normally utilizing sulfur. The tRNA selenium content is neither decreased by the presence of excess sulfur-containing compounds, nor increased by additional selenium in the growth medium. The seleno-tRNAs have been shown to have either lysine or glutamate acceptor specificity. The selenium is present as the selenonucleoside, 5-methylaminomethyl-2-selenouridine (mnm^5se^2U). This nucleoside is the selenium analogue of 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), a naturally occuring nucleoside known to be present at the first position of the anticodon of $tRNA^{Lys}$ and $tRNA^{Glu}$ from E. coli. Analysis of the ribonuclease T_1 oligonucleotides from E. coli selenium containing $tRNA^{Lys}$ indicate that mnm^5se^2U is probably also located at the first position of the anticodon.

This report concerns investigations related to the biosynthesis of the mnm^5se^2U residue in tRNA. The results indicate that mnm^5se^2U is probably a common natural component of E. coli tRNA. Incorporation of selenium is highly specific and appears to involve the direct replacement of sulfur by selenium.

Major Findings

1. Biosynthesis of selenonucleoside under "natural" growth conditions.

E. coli grown on rich, natural media without intentional selenium supplementation were found to synthesize mnm^5se^2U-containing tRNA. For these studies a non-radioactive method of quantitating mnm^5se^2U was required. It was found that as little as 5 pmol of mnm^5se^2U could be quantitated by UV absorbance if the bulk tRNA was hydrolyzed to nucleosides and separated by reversed-phase HPLC while monitoring at 313 nm (the λ max for mnm^5se^2U). Cells grown on beef extract, tryptone-yeast extract, or other meat-based peptones contained from 4 to 61 pmol mnm^5se^2U per A_{260} unit of tRNA. It is anticipated that E. coli in their natural environment (i.e., the human colon) will synthesize various levels of selenium-containing tRNA depending on the level of selenium in the diet. The failure to detect this selenonucleoside in the past during studies of E. coli lysine and glutamate tRNA may have partly been due to the use of purified or minimal media.

2. Levels of mnm^5se^2U and mnm^5s^2U in E. coli tRNA.

The levels of mnm^5se^2U and mnm^5s^2U in E. coli tRNA were measured under a variety of conditions. No mnm^5se^2U and about 150 pmol mnm^5s^2U per A_{260} unit of tRNA were present if selenium was ommitted from the purified minimal medium used. Increasing amounts of mnm^5se^2U and decreasing amounts of mnm^5s^2U were synthesized as the medium selenium concentration increased from 0 to 1 μM. Maximum levels of mnm^5se^2U (60-70 pmol per A_{260} unit tRNA) were given by about 1 μM SeO_3^{2-} or SeO_4^{2-}. Under these conditions the level of mnm^5s^2U was also 60-70 pmol per A_{260} unit, meaning that about 4% of the tRNAs contained mnm^5se^2U and about 4% contained mnm^5s^2U. Higher amounts of mnm^5se^2U were not observed, even if the medium selenium concentration was increased to toxic levels (> 100 μM or if the medium sulfur concentration was decreased to deficient levels (< 40 μM). In all cases the sum of the mnm^5se^2U and mnm^5s^2U levels remained relatively constant at

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120-150 pmol per A_{260} unit. The maximum mnm^5se^2U level was also observed when cultures were grown aerobically or anaerobically, or in rich media supplemented with 1 μM SeO_3^{2-}.

3. Mechanism of selenonucleoside biosynthesis

Modified nucleosides in tRNA arise by the posttranscriptional action of specific enzymes on precursor tRNA substrates. Presumably, the incorporation of selenium and synthesis of the 5-methylaminomethyl side chain of mnm^5se^2U involve a number of enzymatic steps. Details concerning the synthesis of analogous mnm^5s^2U residues in E. coli $tRNA^{Lys}$ or $tRNA^{Glu}$ are unknown except that an S-adenosylmethionine-dependent reaction supplies the terminal methyl group in the 5-methylaminomethyl side chain. A mutant defective in this methylation reaction (obtained from M. G. Marinus) was found to incorporate normal levels of selenium into its tRNA (parent: 56 pmol/A_{260} unit tRNA, mutant: 58 pmol/A_{260} unit tRNA). The selenonucleoside synthesized in the mutant was identified as 5-aminomethyl-2-selenouridine by co-chromatography of its selenium-containing acid degradation product with authentic 5-aminomethyl-2-selenouracil. This new selenopyrimidine was synthesized by Lin Tsai of this laboratory. Although the identity of the 2-thiouridine derivative synthesized by the mutant was not directly determined, it had an elution position during HPLC consistant with the structure 5-aminomethyl-2-thiouridine. The mutant selenonucleoside and thionucleoside could be converted to mnm^5se^2U and mnm^5s^2U, respectively, by incubation of the intact tRNA with S-adenosyl methionine and a crude enzyme preparation from wild-type E. coli. Because a single mutation inhibited methylation of both the seleno-and thionucleoside, the enzyme responsible for this modification apparently lacks specificity for either sulfur or selenium at the 2 position. It is also evident that a complete 5-methylaminomethyl side chain is not required for selenium modification to take place.

Additional studies, designed to further elucidate the nature of the tRNA precursor which serves as the selenium acceptor, have employed chloramphenicol (to increase synthesis of undermodified tRNAs) and rifampicin (to inhibit synthesis of new tRNA). These have indicated (1) that selenium is readily incorporated into pre-formed tRNA, (2) that this selenium-accepting capacity of the tRNA does not readily decrease with time, and (3) that the 5-methylaminomethyl side chain may be either complete or in various stages of completion when selenium modification at the 2 position occurs.

The mechanism of selenium incorporation into mnm^5se^2U is of great interest. It was assumed initially that this process would involve the replacement of oxygen with selenium at the 2 position of a uridine derivative. Several studies, however, indicated that under normal growth conditions there was a substantial pool of pre-formed tRNA molecules which could be modified with selenium to yield mnm^5se^2U residues. Since 5-methylaminomethyl-uridine has not yet been identified in E. coli, it appeared that tRNA containing 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) might be the substrate for selenium modification.

By labeling E. coli during growth with $^{35}SO_4^{2-}$ in the absence of selenium and resuspending the washed cells in fresh medium containing unlabeled SO_4^{2-}, the stability of the ^{35}S label in mnm^5s^2U residues was examined. It was found that label was lost from mnm^5s^2U when SeO_3^{2-} was added to these cultures, but no label

was lost if selenium was ommitted. These data imply that the selenium modification reaction may involve a direct replacement of sulfur with selenium. The mnm^5s^2U containing $tRNA^{Lys}$ and $tRNA^{Glu}$ species normally synthesized in the absence of slenium may be the tRNA substrates for this process.

Initial experiments with a crude, in vitro system (consisting of french pressure cell-fractured E. coli, $^{75}SeO_3^{2-}$, ATP, and Mg^{2+}) have demonstrated that commerical preparations of mnm^5s^2U-containing E. coli $tRNA^{Lys}$ or $tRNA^{Glu}$ can serve as substrates for the modification process. The product of the reaction was mnm^5se^2U. In vitro synthesis of mnm^5se^2U was totally dependent upon the addition of these tRNAs and the presence of ATP. Work with permeablized E. coli cell preparations showed that this ATP-dependent reaction is highly specific for selenium. Large excesses of cysteine or other sulfur-containing compounds did not inhibit. Selenocysteine and SeO_3^{2-} were both effective as selenium sources, whereas selenomethionine was not.

This ATP-dependent, incorporation of selenium to form mnm^5se^2U residues in tRNA is the first in vitro demonstration of an enzymatic activity which specifically acts to sythesize a selenium-containing product. Many enzymes are known which will non-specifically utilize selenium instead of sulfur, and recently an enzyme specific for the degradation of selenocysteine has been described, but no activity had previously been demonstrated in vitro which specifically incorporates selenium into a macromolecule. The existence of specific selenocysteine residues in selenium-requiring enzymes, however, implies that other such activities will be found. It is hoped that further study of the enzymatic incorporation of selenium into tRNA, besides being important in and of itself, may help our understanding of how proteins are specifically modified with selenium.

Proposed Course:

1. The enzymatic incorporation of selenium into tRNA will be further studied. The exact nature of the tRNA and selenium-containing substrates need to be determined. This should allow investigation of how the level of mnm^5se^2U is regulated and why mnm^5s^2U-containing tRNA still persists in the presence of high environmental selenium.

2. The selenium-containing and non-selenium-containing forms of $tRNA^{Lys}$ and $tRNA^{Glu}$ will be purified from E. coli. In collaboration with Wei-Mei Ching of this laboratory, ribosome binding studies will be conducted to determine codon specificity and the efficiency of these tRNAs in protein synthesis will be investigated.

Publications:

Wittwer, A. J.: Specific incorporation of selenium into lysine- and glutamate-accepting tRNAs from Escherichia coli. J. Biol. Chem. 258, 8637-841, 1983.

Ching, W.-M., Wittwer, A. J., Tsai, L., and Stadtman, T. C.: Distribution of two selenonucleosides among the selenium-containing tRNAs from Methanococcus vannielii. Proc. Natl. Acad. Sci. USA 81, 57-60, 1984.

Stadtman, T. C., Ching, W.-M., Hartmanis, M. G. N., Sliwkowski, M., Tsai, L., Wittwer, A. J., and Yamazaki, S.: Bacterial selenoenzymes and seleno-tRNAs: Studies on chemical composition and structure. Proceedings of the 4th International Conference on the Organic Chemistry of Selenium and Tellurium. Birmingham, England, Univ. of Aston, 1983, pp. 521-530.

Wittwer, A. J., Tsai, L., Ching, W.-M., and Stadtman, T. C.: Identification and synthesis of a naturally occuring selenonucleoside in bacterial tRNAs: 5-methyl-aminomethyl-2-selenouridine. Biochemistry, 1984, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00246-03 LB

PERIOD COVERED

October 1, 1983, September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of Selenium-containing Enzymes form Clostridium kluyveri

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mark X. Sliwkowski, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several selenium-containing proteins of bacterial origin are being investigated. Thiolase and 3-hydroxybutyryl-CoA dehydrogenase from Clostridium kluyveri contain selenium as selenomethionine. Detailed studies have been conducted on the localization and distribution of selenium throughout the primary structure of these enzymes. Peptide mapping experiments using reverse phase HPLC were performed with the purified enzymes labelled with ^{75}Se and ^{35}S. The results of these studies indicated that selenium was distributed throughout the primary structure of thiolase and 3-hydroxybutyryl-CoA dehydrogenase in a manner that paralleled methionine. Analyses of proteolytic digests of these proteins, support a nonspecific incorporation mechanism of selenium. However, this mechanism appears to be limited to selenomethionine substitution for methionine since no selenium incorporation as selenocysteine was detected. Selenoprotein A of the clostridial glycine reductase complex which contains selenium as selenocysteine is also being investigated. An improved method for the purification of selenoprotein A from both Clostridium sticklandii and Clostridium purinolyticum has been developed using an ion exchange HPLC step. Milligram quantitites of this protein have now become available for structural studies. In contrast to thiolase and 3-hydroxybutyryl-CoA dehydrogenase, selenium has been found to be localized at a specific residue within the polypeptide chain. A tryptic peptide has been isolated by reverse phase HPLC which contains the selenocysteine residue. The peptide has been partially sequenced and also appears to contain the two cysteine residues of the protein. The selenocysteine residue however, occurs in a unique position near the carboxylterminus of the protein as shown with carboxypeptidase Y digestion.

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Project Description:

Objectives:

The ultimate goals of this research have been to determine the mechanism(s) by which selenium is incorporated into proteins and to investigate the role of the selenium moiety in catalysis. A unique form of selenium, that of selenomethionine, has been indentified in two enzymes, thiolase and 3-hydroxybutyryl-CoA dehydrogenase from Clostridium kluyveri. Preliminary evidence has indicated that the mechanism of selenium incorporation in these enzymes is fundamentally different from those proteins which contain the element as selenocysteine. A selenocysteine-containing protein, selenoprotein A of the clostridial glycine reductase complex, is also being used as a model for selenium incorporation. A peptide, which contains the selenocysteine residue, has been purified, characterized, and partially sequenced. Thus, much of the ground work necessary to study selenium function at the molecular level is nearing completion.

Major Findings:

(1) Clostridium kluyveri incorporates selenium as selenomethionine into its acetoacetyl-CoA thiolase when grown in media containing normal sulfur-to-selenium ratios. Antibodies raised against the purified enzyme permitted quantitiative immunoprecipitation of thiolase from crude cell extracts and thus facilitated the systematic analysis of effects of wide variation in sulfur-to-selenium ratios on selenium incorporation into the enzyme. The extent of incorporation of selenium into thiolase was found to be dependent on the form of selenium supplied. When [^{75}Se]selenomethionine was the source of selenium, the incorporation of selenium into thiolase was inversely proportional to the level of added methionine. However, similar levels of methionine failed to decrease the incorporation of selenium from selenite. To study the location of selenomethionine and methionine residues in the polypeptide chain of the enzyme, thiolase was prepared from cells cultured in the presence of $H_2{}^{35}SO_4$ or $Na_2{}^{75}SeO_3$. Purified thiolase, labeled with ^{35}S or ^{75}Se, was subjected to extensive peptide mapping by reverse phase HPLC. (Similar studies were also conducted with 3-hydroxybutyryl-CoA dehydrogenase.) The peptide maps of these enzymes indicated that selenium was distributed throughout the primary structure in a manner that paralleled methionine. A curious feature of this mechanism however, is that none of the selenium appears as selenocysteine residues. Prokaryotes and also yeasts and some higher plants appear to have the capacity of converting excess selenium into selenomethionine, which can then be readily translated into proteins in place of methionine. The possibility that this mechanism of selenium storage occurs in mammals has yet to be established. For the most part, this project has now been completed and we have turned our attention to those proteins which contain selenocysteine.

(2) The existence of a specific selenocysteine-tRNA, which recognizes a specific codon, has been postulated to account for selenium incorporation into glutathione peroxidase. However, other studies with this enzyme have suggested that this is not the case. In light of our current studies on bacterial selenoenzymes, we feel that selenium incorporation most likely occurs posttranslationally. Covalent modification of an existing amino acid within the holoprotein is envisioned, with subsequent insertion of selenium. Thus, all selenoproteins may share some structural features which are recognized by the specific enzyme(s)

involved in the modification.

To test this hypothesis, we have embarked on a series of experiments with selenoprotein A of the clostridial glycine reductase complex. Recently, we have developed an improved method for the purification of selenoprotein A, which yields milligram quantitites of the protein in a few relatively simple steps. This isolation procedure has been used with two different organisms, Clostridium sticklandii and Clostridium purinolyticum. The key improvement in this method has been the addition of an HPLC ion exchange step. These columns, which are synthetic resins rather than silica based have been used by several other people in our laboratory with similar success.

(3) A tryptic peptide, from selenoprotein A, which contains the selenocysteine residue has been isolated by reverse phase HPLC and partially sequenced. The peptide, which constitutes nearly 25% of the selenoprotein A sequence, appears to be derived from the C-terminal region of the protein. In addition to containing selenocysteine, this peptide also contains both of the cysteine residues of the protein.

Recently Dr. Levine has introduced a precolumn o-phthaldialdehyde procedure for amino acid analysis to the laboratory. This method is a superb technique for analyzing alkylated derivatives of selenocysteine, and has been used with hydrolysates of purified selenoprotein A. Also, since the analysis procedure is very rapid (~ 12 min per run), end terminal analyses of proteins and peptides are easily performed. Total release of the selenium-containing amino acid is seen with carboxypeptidase Y digestion of selenoprotein A. This is further indication that the selenium moiety is near the carboxyl-terminus.

Proposed Course:

We are in the process of making antibodies to selenoprotein A. Previous studies have indicated that this protein is not very antigenic, perhaps because of its small size (M_r= 12,000). To circumvent this problem, we will conjugate the selenoprotein to a carrier protein, probably bovine serum albumin. These antibodies can then be used for a series of in vitro translation experiments. By translating the clostridial message exogenously, we hope to gain insights into a possible precursor from of selenoprotein A.

As mentioned earlier, sequence studies with selenopreotein A have been very successful. In the next phase of our project, we plan to clone the gene which codes for selenoprotein A. Clones will be screened with antibody and if these experiments are successful, we hope to use DNA sequencing techniques to determine the complete sequence of the protein. Information from these studies, coupled with the results of the peptide sequencing, should provide an unambiguous answer to the nature of the selenoprotein A precursor, both from the protein and DNA level. Additionally, the cloned selenoprotein A will provide a readily available source of substrate material, for studies aimed at identifying the enzymes and factors which are required for selenium incorporation.

Publications:

Sliwkowski, M. X.: Characterization of selenomethionine in proteins. Meth. Enzymol. 107, 620-623, 1984.

Sliwkowski, M. X. and Hartmanis, M. G. N.: Single step simultaneous purification of thiolase and 3-hydroxybutyryl-CoA dehydrogenase from Clostridium kluyveri. Anal. Biochem., 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00247-03 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Proteolysis of Glutamine Synthetase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Jo Ellen Roseman, Guest Worker, Laboratory of Biochemistry, NHLBI

Others: E. R. Stadtman, Chief, Laboratory of Biochemistry, LB, NHLBI
R. L. Levine, Senior Investigator, LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The turnover of Escherichia coli glutamine synthetase occurs in two steps. First, the glutamine synthetase is oxidatively modified, rendering it enzymatically inactive; and second, the modified glutamine synthetase is degraded. The oxidation step, catalyzed by several mixed function oxidase systems, results in the loss of a single histidine and the generation of a carbonyl group. We proposed that the oxidation step marks glutamine synthetase for degradation. The purpose of this project is to isolate and characterize the protease activity which degrades the modified glutamine synthetase. A protease that degrades the modified GS 10-20 times more rapidly than native has been purified from E. coli extracts. The protease precipitates in a 60-70% ammonium sulfate cut, does not bind to DEAE ion exchanger at pH 7.5, binds to an HPLC Phenyl column, and to an HPLC DEAE column at pH 9. The purified protease migrates on HPLC gel chromatography as a single protein peak with a molecular weight of 70,000-80,000. The protease activity is optimum at pH 9, and its isoelectric pH is 7-7.4. It is activated 2-4 fold by 50 μM zinc plus 1 mM magnesium. The protease is inhibited by aprotinin but not by other serine protease inhibitors. Metal chelators and thiol reagents also inactivate the protease, and these effects are not reversible.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: To elucidate the mechanism of intracellular protein turnover, we have been attempting to isolate from bacteria a system that will degrade normal cell proteins. Studies originally done in 1977 showed that glutamine synthetase (GS) activity is lost in nitrogen-starved bacterial cells prior to loss of GS cross-reacting protein. This led to the hypothesis that a modification which results in inactivation provides a signal to the proteolytic machinery of the cell. The inactivating activity found in bacterial extracts can be mimicked by a model system, consisting of iron, oxygen, and ascorbic acid. Escherichia coli extracts preferentially degrade purified, ascorbate modified GS as compared to native GS. The ascorbate modified GS has been shown to be similar to native GS: it comigrates on native and SDS polyacrylamide gels, has the same fluorescence emission spectrum and shows only subtle differences in its UV absorption spectrum. The ascorbate inactivated GS shows the loss of a single histidine residue per 50,000 molecular weight subunit and the addition of a carbonyl group.

The goal of this project is to purify a proteolytic activity that preferentially degrades the ascorbate-modified GS over the native, presumably by recognition of the subtle and specific modification imparted by ascorbate treatment.

Methods Employed:

The approach we have taken is to use a known physiologic substrate and try to find an E. coli protease that will degrade it. By growing cells on radioactive nutrients and allowing them to incorporate the nutrients into protein, we have produced radioactive protein that is unmodified by isotopic labeling techniques.

GS was purified by the zinc precipitation method from an overproducing E. coli K12 strain grown on radioactive amino acids. The purified GS was of high enzymatic specific activity and was uniformly labeled with ^{14}C-amino acids. A portion of this native enzyme was inactivated by the ascorbate model inactivating system.

The radioactive GS is insoluble in trichloroacetic acid, and we developed an assay to measure the production of trichloroacetic acid soluble radioactivity as a result of protease treatment.

Purification of the protease has employed fractional precipitation, conventional as well as HPLC ion exchange, hydrophobic and gel chromatography, as well as dye affinity chromatography.

Major Findings:

Starting from E. coli whole cell extracts, a proteolytic activity has been purified which degrades oxidized GS ten times more rapidly than the native. The protease precipitates in a 60-70% ammonium sulfate cut, does not bind to DEAE ion exchanger at pH 7.5, binds to HPLC Phenyl in 1M ammonium sulfate and binds to HPLC DEAE at pH 9. Following purification using these steps, the protease chomatographs on HPLC gel chromatography as a single protein peak of molecular weight 70,000-80,000.

The purified protease is inhibited by aprotinin but not by other serine protease inhibitors. Chelating agents and thiol reagents inhibit the protease, and these effect are not reversible. The alkylating agent ethyleneimine also permanently inactivates the protease. The protease has an alkaline pH optimium and its isoelectric pH is in the neutral range.

The protease also binds to Blue B matrex gel. The unbound fraction, which contains no protease activity stimulates the eluted protease 2-4 fold. The unbound stimulatory component is heat stable and dialyzable, and binds to Chelex 100. Following purification on Chelex 100, the activating component was analyzed by atomic absorption and found to contain zinc and magnesium. Addition of Zn plus Mg to the protease, at levels comparable to levels found in the activating component, fully replaces its stimulatory effects.

The ability of the protease to recognize other modifications of glutamine synthetase was examined. The protease degrades glutamine synthetase in which tyrosine residues are nitrosylated, as well as glutamine synthetase which is denatured by urea.

Proposed Course:

Several properties of glutamine synthetase degradation by the protease will be examined including kinetic parameters, extent of degradation, and characterization of the products of the reaction. In addition, its potential capacity to degrade other modified enzymes will be explored.

Publications:

Oliver, C. N., Fulks, R., Levine, R. L., Fucci, L., Rivett, A. J., Roseman, J. E., and Stadtman, E. R.: Oxidative inactivation of key metabolic enzymes during aging. In Roy, A. K. and Chatterjee, B. (Eds.): Molecular Basis of Aging. New York, Academic Press, 1984, pp. 237-264.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00248-02 LB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Oxidative Modification in Protein Turnover in Mammalian Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. Jennifer Rivett — Visiting Fellow — LB, NHLBI

Others: E. R. Stadtman, — Chief, Laboratory of Biochemistry — LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mixed-function oxidation of Escherichia coli glutamine synthetase results in the modification of a single histidine residue per subunit, introduction of a carbonyl group, and loss of catalytic activity. This oxidative inactivation results in increased susceptibility to proteolytic attack.

Four proteases which preferentially degrade the oxidized form of [^{14}C]-labeled glutamine synthetase have been purified from liver. One of the proteases was identified as cathepsin D which is of lysosomal origin. The other three proteases were present in the cytosol. Two of them required calcium for activity; the other did not. The latter protease, a thiol protease referred to simply as the alkaline protease, has been purified to apparent homogeneity from mouse and rat liver acetone powders. Its molecular weight, determined by gel filtration, was around 270,000. Multiple bands of molecular weight 25,000-32,000 were obtained on SDS gels. Native glutamine synthetase was not significantly degraded by the cytosolic proteases at physiological pH values. Oxidative inactivation of the enzyme caused a very dramatic increase in susceptibility to attack by these proteases. Adenylylation, which causes reversible loss of glutamine synthetase biosynthetic activity, had little effect on its rate of degradation by any of the proteases. Proteolysis was increased following relaxation or dissociation of the enzyme.

The characteristics of the oxidative modification of rabbit muscle enolase are similar to those of E. coli glutamine synthetase. The oxidized form of this enzyme was also preferentially degraded by the purified liver alkaline protease. The ability of cytosolic proteases to specifically recognize the oxidized form of glutamine synthetase and enolase suggests that oxidative modification of proteins may be involved in intracellular protein turnover.

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Project Description:

Objectives: Previous studies in this laboratory have suggested that oxidative modification of bacterial glutamine synthetase marks the enzyme for degradation. The main objective of this study was to investigate the possible role of mixed-function oxidation of proteins in intracellular protein turnover.

Methods Employed:

1. [^{14}C]Glutamine synthetase (GS) was prepared by growing an overproducing strain of E. coli on [^{14}C]amino acids.

2. Purified [^{14}C]GS was oxidatively modified by a mixed-function oxidation system consisting of ascorbate, $FeCl_3$, and oxygen.

3. Protease activity was measured by the release of trichloroacetic acid soluble products from native or oxidized [^{14}C]GS.

4. Proteases were purified from rat or mouse liver using oxidized [^{14}C]GS as substrate. The proteases were (a) cathepsin D, (b) the calcium-dependent proteases, calpain I and II, and (c) the alkaline protease.

5. Other enzymes including enolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and ornithine decarboxylase were oxidatively inactivated by the ascorbate system.

6. Proteolysis of unlabeled proteins was assayed by fluorescamine determination of trichloroacetic acid soluble products.

Major Findings:

1. Preferential degradation of the oxidatively modified form of GS -- There are intracellular mammalian proteases which recognize oxidized GS. The nonlysosomal proteases, the calcium-dependent proteases, and the alkaline protease do not degrade native GS. The lysosomal protease, cathepsin D, and other proteases such as trypsin and papain do degrade the native enzyme, but the oxidized form is degraded more rapidly.

2. Effect of other GS modifications on rate of degradation -- Adenylylation has no effect on the susceptibility of GS to proteolysis. Relaxation of the GS (by addition of EDTA) or dissociation of subunits causes a dramatic increase in proteolysis of native GS and also increases the rate of degradation of the oxidatively modified form.

3. Characteristics of the modified GS which cause increased susceptibility to proteolysis -- Using the ascorbate system, loss of GS catalytic activity precedes the increase in susceptibility to proteolytic attack.

4. Oxidative modification of other proteins: Effect on proteolysis -- The characteristics of the oxidative inactivation of rabbit muscle enolase and of the subsequent increase in susceptibility to proteolysis by the liver alkaline protease and trypsin are similar to those of E. coli GS.

5. Purification and properties of the alkaline protease -- The alkaline protease has been purified to a single band on polyacrylamide gels from rat and mouse liver acetone powder. It has a molecular weight around 270,000 determined by gel filtration. Multiple bands with molecular weights varying from 25,000-32,000 were obtained on SDS polyacrylamide gels. The protease is inhibited in crude liver preparations. Results with purified immunoglobulin fractions of rabbit antiserum raised against the alkaline protease suggest that there are differences in the protease isolated from acetone powder and that from fresh liver.

6. Protease levels in old rat liver -- The proteases which recognize oxidatively modified GS are present in old (31 months) rat liver at apparently similar levels to those found in livers from younger (9 months) rats. However, because of the large amount of inhibitory activity (competing protein or endogenous protease inhibitors) present in crude preparations, measurement of protease activity, even after partial purification may not be an adequate method of comparing protease levels.

Significance to Biomedical Research and the Program of the Institute:

Intracellular proteins are continuously turning over with widely differing half-lives. The mechanism of control of the degradation process, its apparent energy requirement, its link with protein synthesis, and its rapid response to the nutritional state of the cell are not understood. In mammalian cells, proteins can be degraded by both lysosomal and nonlysosomal systems. The nonlysosomal system is of particular interest since it is thought to account for most of the intracellular protein degradation under normal conditions. The long-term aim of this study is to help elucidate the mechanism of protein turnover in mammalian cells. It relates to the following areas of interest:

1. Selection of proteins for degradation -- The degradation of proteins may be regulated by a two-step mechanism in which the protein is first modified such that it becomes more susceptible to proteolytic attack. Mixed-function oxidation is one possible method of marking proteins for degradation. Others include phosphorylation, deamidation, conjugation with ubiquitin, sulfhydryl modification.

2. Characteristics of the proteases -- Most of the studies on intracellular proteases have involved the low molecular weight lysosomal proteases. Much less is known about cytosolic proteases at least some of which are higher molecular weight, multisubunit enzymes.

3. Activation of proteases -- Cytosolic proteases may require activation by an effector/substrate or by removal of an inhibitor. The presence of specific intracellular protease inhibitors has been demonstrated, but very little is known of the mechanism by which the protease/inhibitor interaction is controlled.

4. What features of a protein are recognized by proteases and how is it degraded?

5. Accumulation of inactive enzyme forms during aging -- Oxidative modification has also been implicated in the aging process. Many of the enzymes which can be inactivated by oxidative modification have been shown by others to accumulate as inactive or less active forms during aging. One possible explanation for

this accumulation is a defect in the degradation process. It was therefore of interest to investigate the possibility of changes in protease levels during aging.

Proposed Course:

1. Further characterization of the alkaline protease - inhibitor specificity, nature and function of subunits, comparison of protease isolated from acetone powder and from fresh liver, nature of inhibition in crude liver preparations.

2. Investigation of the mechanism of degradation of oxidized GS -- Changes in the GS molecule which cause enhanced susceptibility to proteolysis, intermediates/products of the degradation by the alkaline protease.

3. Studies of the susceptibility to proteolysis of other oxidized proteins.

Publications:

Oliver, C. N., Fulks, R., Levine, R. L., Fucci, L., Rivett, A. J., Roseman, J. E. and Stadtman, E. R.: Oxidative inactivation of key metabolic enzymes during aging. In Roy, A. K. and Chatterjee, B. (Eds.): Molecular Basis of Aging. New York, Academic Press, 1984, pp. 237-264.

Rivett, A. J., Roseman, J. E., Oliver, C. N., Levine, R. L., and Stadtman, E. R.: Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. In Khairallah, E. A., Bond, J. S., and Bird, J. W. C. (Eds.): Intracellular Protein Catabolism, New York, Alan R. Liss, 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00252-02 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Activation of the Mg(II)-ATP-dependent Phosphoprotein Phosphatase (F_c)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stewart R. Jurgensen	Staff Fellow	LB, NHLBI
Others:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
	Charles Y. Huang	Research Chemist	LB, NHLBI
	Emily Shacter	Staff Fellow	LB, NHLBI

COOPERATING UNITS *(if any)*

Jackie Vandenheede, Katholieke Universitiet te Leuven, Belgium
Susan Taylor, University of California, San Diego

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous work (see last year's annual report) involved study of the mechanism of activation of the Mg(II)-ATP-dependent phosphoprotein phosphatase (termed F_c-M) isolated from rabbit skeletal muscle. This phosphatase appears to be a major phosphatase in rabbit skeletal muscle and plays an important role in regulating glycogen synthesis and degradation. F_c·M is inactive as isolated, and is activated by kinase F_A and Mg(II)-ATP. There have been conflicting reports on whether the catalytic subunit of cAMP-dependent protein kinase is able to substitute for kinase F_A in the phosphatase activation reaction. We find that cAMP-dependent protein kinase (in the presence of cAMP and Mg(II)-ATP) is unable to activate the phosphatase. However, inclusion of cyclic A kinase in the activation reaction with kinase F_A results in a cyclic AMP-dependent inhibition of the phosphatase. We have shown that the phosphatase inhibition is due to the dissociated regulatory subunit of the type II cAMP-dependent protein kinase (R_2^{II}). The isolated protein kinase catalytic subunit (C) does not affect the kinase F_A and Mg(II)-ATP activated phosphatase. Inhibition of the phosphatase by isolated R_2^{II} can be reversed by the addition of an excess of C to reform the protein kinase holoenzyme R_2C_2. The addition of cAMP to dissociate the holoenzyme restores the phosphatase inhibition. Heating R_2^{II} for 5 minutes at 90°C destroys both its ability to inhibit the kinase activity of isolated C and its inhibitory effect on phosphatse F_c·M. Further work was directed at characterizing the inhibition of phosphatase by R_2^{II}. R_2^{II} inhibition of phosphatase F_c·M appears to result from both a direct effect on the activated phosphatase and an inhibitory effect on phosphatase activation.

Project Description:

Objectives: This project has been aimed at gaining an understanding of the mechanism of activation of the Mg(II)-ATP-dependent phosphoprotein phosphatase (F_c·M), and the regulation of its activity. Our work has implicated phosphorylation/dephosphorylation of the modulator component of F_c·M in the interconversion of active and inactive phosphatase. An important aspect of this project has been an investigation of the inhibition of phosphatase activity that we observed when cAMP and cAMP-dependent protein kinase are included in the phosphatase activation reaction. We initially suspected that this inhibition might be due to a phosphorylation by protein kinase at an inhibitory site on the F_c·M complex. However, after further investigation, we have shown that this inhibition is due to the dissociated regulatory subunit (R_2^{II}) of cAMP-dependent protein kinase. Inhibition of phosphatase activity upon activation of cAMP-dependent protein kinase may play an important regulatory role in phosphorylation/dephosphorylation cascade systems involving this kinase and phosphatase.

Major Findings:

The regulatory subunit of type II cAMP-dependent protein kinase is shown to be a potent inhibitor of the phosphatase F_c·M. Phosphatase inhibition by R_2^{II} is not just a simple inhibition of the activated phosphatase. Addition of R_2^{II}, after phosphatase activation, results in less inhibition compared to when R_2^{II} is added just before initiating F_c·M activation. When kinase F_A levels are decreased during activation of F_c·M, lower apparent steady state levels of phosphatase activity occur. The degree of phosphatase inhibition by R_2^{II} is greater at a lower concentration of kinase F_A compared to when a maximally activating concentration of kinase F_A is used. However, the inhibitory activity of R_2^{II} is not affected by preincubation with kinase F_A and Mg(II)-ATP, indicating that under the conditions used for phosphatase activation, kinase F_A is not converting R_2^{II} to a less inhibitory form (for example by a phosphorylation reaction). Phosphatase inhibition curves as function of the concentration of R_2^{II} have been determined under several different conditions. The kinetic pattern of inhibition has also been determined to be noncompetitive with respect to substrate phosphorylase a. These results, and the fact that R_2^{II} inhibits even with phosphorylase a in large excess over R_2^{II} concentration, demonstrates that R_2^{II} is not inhibiting the phosphatase by a substrate directed effect nor by acting as a substrate analog. The time course of phosphatase activation by kinase F_A and Mg(II)-ATP has been determined in the presence and absence of added R_2^{II} and cAMP. The degree of phosphatase inhibition by R_2^{II} is greater at earlier points in the phosphatase activation curve and appears to decrease, approaching a final level of inhibition as an apparent steady state level of phosphatase activity is achieved. The transient nature of the inhibitory capacity of R_2^{II} towards phosphatase F_c·M may be a feature of regulatory importance and requires further investigation.

cAMP-dependent protein kinase is known to act at several points in the glycogen phosphorylase/glycogen synthase cascade, leading to increased phosphorylation and activation of phosphorylase and phosphorylation and inactivation of synthase. Enhanced sensitivity may be achieved by simultaneous inhibition of phosphatase activity upon activation of cAMP-dependent protein kinase. This effect may also be multiplied due to the fact that phosphatase F_c·M is a type 1

phosphatase, of broad substrate specificity, which dephosphorylates many of the phosphorylation sites of the phosphoproteins involved in this system. Furthermore, the coincident activation of protein kinase activity and inhibition of protein phosphatase activity reduces the ATP consumption of this system making it more energetically efficient.

Proposed Course:

We plan to pursue several lines of investigation regarding the activation and regulation of the Mg(II)-ATP-dependent phosphoprotein phosphatse (F_c·M). We will compare the type I regulatory subunit of cAMP-dependent protein kinase with the type II regulatory subunit in their ability to inhibit phosphatase F_c·M. It is known that R_2^{II} can be phosphorylated at several different sites by different protein kinases. In future work, we would like to address the question of whether phosphorylation at any of these sites affects the inhibition of phosphatase F_c·M. Since R_2^I is reported not to be phosphorylated by these same kinases and it also lacks the "autophosphorylation" site at which R_2^{II} is phosphorylated by the catalytic subunit, comparison of inhibition by R_2^I and R_2^{II} may be suggestive of whether or not regulatory subunit phosphorylation affects phosphatase inhibition. It is also of interest to obtain direct evidence of binding of R_2^{II} (R_2^I) to the phosphatase.

Recently, Vandenheede and colleagues have obtained evidence for a change in conformation of the catalytic subunit upon thiophosphorylation of the modulator subunit of F_c·M. We and others have observed that when ATPγS is substituted for ATP, there is no activation of the phosphatase by kinase F_A although thiophosphate is incorported into the modualtor component of F_c·M. We plan to compare the site(s) of thiophosphorylation and phosphorylation of the modulator component of F_c·M.

We also plan to explore the use of HPLC in the isolation of the phosphatase F_c·M and of the modulator protein (I-2). The newer anion exchange HPLC resins may be very useful in isolation of F_c·M. The enzyme isolated in this laboratory and in the laboratory of Vandenheede et al. appears to have an ~ 62,000 Da polypeptide associated with it. New purification techniques may allow separation and reconstitution of the enzyme with regard to this component. This will be helpful in determining whether the 62,000 Da polypeptide is truly part of this enzyme and what its function might be (for example, is it involved in the inhibition by R_2^{II}?) Reverse phase HPLC may also be of use in the isolation of modulator (I-2) since this polypeptide is known to be both heat stable and stable to acid precipitation by trichloroacetic acid.

Publications:

Jurgensen, S., Shacter, E., Huang, C. Y., Chock, P. B., Yang, S.-D., Vandenheede, J. R., and Merelevede, W.: On the mechanism of activation of the ATP·Mg(II)-dependent phosphoprotein phosphatase by kinase F_A. J. Biol. Chem. 259: 5864-5870, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00254-02 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection of Protein Tyrosine Residues Linked by Nucleotide Phosphodiesters

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Todd M. Martensen Guest Worker LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.12	0.1	0.02

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nucleotide linked to protein through phosphodiester bonds has not been found in normal eucaryotic cells. A sensitive detection methodology for this post-translational modification may be possible by the measurement of tyrosine phosphate which is produced by micrococcal nuclease treatment of nucleotidylylated protein. Quantification of tyrosine phosphate before and after enzymatic treatment can be used to detect phosphodiester linkages of nucleotide to protein. Studies on the ability of a nuclease to remove adenosine from adenylylated glutamine synthetase found that the native protein is resistant to nuclease atack while the denatured form readily undergoes deadenosylation. This may indicate that posttranslational modification sites of proteins are protected from nonspecific enzymatic attack by tertiary or quaternary structural factors which may be involved in the control of enzymatic interconversion of proteins. Nucleotide specificity requirements were studied for the adenylylation of glutamine synthetase with [α-^{32}P]ATP [α-^{32}P]AMPPNP and [α-^{35}S]thiophosphate derinatives of ATP. The α-thiophosphorylated ATP but not AMPPNP was utilized as a substrate. It was not possible with the deadenylylating enzyme to cleave the thiophosphodiester bond to glutamine synthetase.

Snake venom phosphodiesterase binds adenylylated glutamine synthelase ~ 1000-fold tighter than a small molecular weight substrate, nitrophenyl dTMP(5'). The tighter binding is inversly correlated with catalysis.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives: Linkage of nucleotides to protein tyrosine residues by phosphodiester bonds has been extensively studied in the regulation of glutamine synthetase and a regulatory protein, P_{II}, which modulates the activity of the interconverting enzyme, ATase. Characterization of putative nucleotide phosphodiester bonds to protein tyrosine residues in eucaryotic cells requires assays to detect this novel posttranslation modification. This modification has been demonstrated to result after poliovirus infection of mammalian cells, but it is not known to occur naturally. The development of sensitive assays to detect these modifications would allow the scope of the modification to be investigated. Since these linkages are present in two proteins of E coli, adenylylated glutamine synthetase and uridylylated P_{II}, these proteins can be used to develop detection methods, and the interconverting enzymes substrate specificities can be analyzed.

Major Findings:

Three techniques can be used for detection of protein tyrosine nucleotide phosphodiester bonds to [^{32}P, ^{14}C]-nucleotide: (I) Treatment with snake venom diesterase will release ^{32}P and ^{14}C into the supernatant after protein is reprecipitated with TCA. (2) Treatment with micrococcal nuclease will release ^{14}C but not ^{32}P into the supernatant after precipitation. The specificity of the nuclease will produce at [^{32}P]Tyr-P residue at the nucleotide ligation site. (3) Base hydrolysis of nucleotide ligated to tyrosine through a 5'-phosphodiester linkage produces Tyr-P in 20% yield (in adenylylated glutamine synthetase). Tyrosine O-AMP has not been found and is probably not stable to complete base hydrolysis. Measurement of protein tyrosine kinase activity in EAT cell extracts with [γ-^{32}P]Tyr-P arises from transfer of the γ-phosphoryl bond of ATP to a protein tyrosine residue. The possibility that some proportion of the Tyr-P is derived from a [^{32}P]tyrosine O-nucleotide bond which arose from transfer of the ^{32}P group to the α position of a nucleotide was checked. Significant amounts of [^{32}P]Tyr-P were found when cell extracts incubated with [α-^{32}P]ATP (for 5 minutes at 37°C) were precipitated with TCA and the precipitate analyzed for Tyr-P. Whether the [^{32}P]Tyr-P formed arose from phosphodiester bonds or monoester bonds (from transfer of ^{32}P to the gamma position of ATP by enzymatic activities in cell extracts) remains unresolved. To prevent release of the γ-phosphoryl bond of ATP, the ATP analog AMPPNP was used to determine whether it would serve as a substrate for the adenylyltransferase (ATase) of E coli. AMPPNP (α-^{32}P) was neither utilized by the ATase as a nucleotide substrate for the adenylylation reaction nor did it prevent the adenylylation of glutamine synthetase by [α-^{32}P]ATP at 100-fold higher concentrations. Another nucleotide substrate was used for the ATase which contained a thiophosphoryl group at the α position of ATP. [^{35}S]adenosine-5'-(α-thio)triphosphate was utilized yielding the nucleotide [^{35}S]thiophosphotediester of glutamine synthetase. The reaction required rate was slow vs ATP, and the yield of thiophosphodiester adenylylated glutamine synthetase appeared to plateau when 7 of 12 residues of the dodecamer were modified. The modified protein displayed the Mn^{2+} requirement that is characteristic of normal adenylylated glutamine synthetase modified to the same extent. The thiophosphodiester modified glutamine synthetase was neither deadenylylated by the ATase in the deadenylylation reaction nor did it appear to inhibit the deadenylylation of adenylylated glutamine synthetase.

Previously it was found that denatured carboxymethylated adenylylated glutamine synthetase (CMGS-AMP) was deadenosylated by micrococcal nuclease while the native enzyme (GS-AMP) was resistant. To determine whether there was a similar difference in the ability of snake venom diesterase (SVD) to cleave the phosphodiester bond, both CMGS-AMP, GSAMP, and a small molecular weight compound p-nitrophenyl 5'-dTMP (PNPTMP) were utilized as substrates. Little difference was found in the ability of SVD to cleave CMGS-AMP and GS-AMP; the denatured substrate was hydrolyzed at a somewhat faster rate in contrast to the findings with micrococcal nuclease. Considerable difference was seen in the kinetic properties of CMGS-AMP vs PNPTMP, however. Under identical reaction conditions, the K_m values were 39 mM with PNPTMP and 28 nM with CMGS-AMP. The V_m values were 5.1 µmol/min/mg for PNPTMP and 32 nmol/min/mg for GM GS-AMP. The increased binding of the macromolecular substrate is inversly related to the catalytic rate which may indicate the macromolecular substrate slowly dissociates from the catalyst or bends in such a manner as to inhibit the mobility of catalytic groups of the SVD.

Significance to Biomedical Research and the Program of the Institute:

The ligation of nucleotide to protein is known to indicate either specific viral infection or enzyme regulation. The discovery of these modifications in eucaryotic cells would be useful to understand how certain types of viruses infect cells, and uncover unique new species of heretofore unkown covalent protein modifications.

Proposed Course:

Further studies of the model protein adenylylated glutamine synthetase and uridylyated P_{II} by different cleavage methods will be done to try to detect O-nucleotidyl tyrosine on the amino acid analyzer. Studies of base hyrolysates of cell extracts incubated with [^{32}P]α-ATP will be continued to determine the nature of the phosphoryl bond. If the phosphodiester bonds to tyrosine are formed in extracts incubated with alpha labeled ATP, treatment of aliquots of a solubilized TCA precipitate with the two nucleases followed by quantification of the [^{32}P]Tyr-P (after base hydrolysis of the digest) may provide sufficient evidence. Of the ^{32}P in phosphodiester bonds to tyrosine, 20% will be recovered after hydrolysis of the untreated aliquot; the venom nuclease treated sample will yield no [^{32}P]Tyr-P and the micrococcal nuclease treated sample will yield four times the quantity of Tyr-P in the untreated sample (the monoester is recovered in 80% yield).

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00255-01 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis of Cofactors & Amino Acid Components of Protein in Anaerobic Bacteria

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Edward DeMoll Staff Fellow LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(1) Selencysteine derives from alanine in protein A of Clostridium purinolyticum glycine reductase.

(2) Guanosine is incorporated almost completely into the deazaflavin cofactor, F_{420}, of Methanococcus vannielii, thereby providing half of the atoms of the three-ring structure and the ribose side chain.

Project Description:

The mechanism of biosynthesis of selenocysteine is not known. Two questions need to be answered to solve its metabolic origin:

(1) From what precursors do the atoms in selenocysteine arise?

(2) Is the molecule synthesized before incorporation into protein or is some other amino acid modified posttranslationally?

Major Findings:

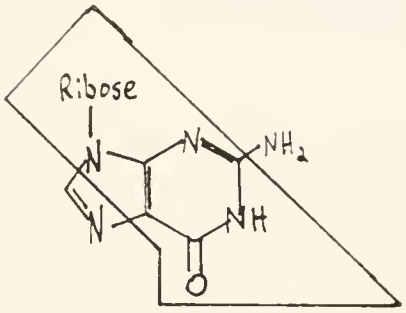
(1) Tritium was incorporated into selenocysteine isolated from glycine reductase protein A of Clostridium purinolyticum grown in the presence of L-[^{3}H]alanine. Tritium was not detected at significant levels in any other amino acid. This was tested in three different chromatography systems, two ion-exchange and one pre-column derivitization reverse phase. In each case, tritium coeluted with selenocysteine and alanine.

Project Description:

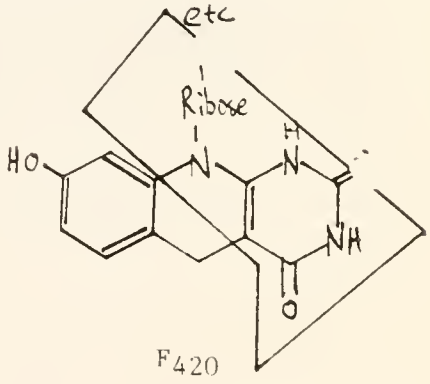
Objective: To elucidate the biosynthetic pathway of the 8-hydroxy-5-deazaflavin cofactor from Methanococcus vannielii.

Major Findings:

(2) Uniformly labeled ^{14}C-guanosine was incorporated into the deazaflavin cofactor (F_{420}) of growing M. vannielii to a specific activity of approximately 30% of the exogenously supplied substrate. Presumably the dilution of specific activity was due to endogenous F_{420} biosynthesis. Preliminary evidence indicates that in this conversion, at least the indicated atoms are transferred:



Guanosine



F_{420}

Proposed Course of Action:

(1) a. The role of alanine as a precursor to selenocysteine is to be clarified by obtaining more accurate measurements of specific activity of selenocysteine synthesized from L-[^{3}H] alanine. Determination of the

mechanism of this reaction may require mass spectra of selenocysteine synthesized from L-[^{2}H] alanine.

b. It is also necessary to determine whether this reaction is posttranslational or not.

(2) It is planned to determine the metabolic origins of all of the atoms in the F_{420} cofactor. This should include examination of the enzymology in as many reactions as is feasible.

Publications:

DeMoll, E., White, R. H., Shive, W., Determination of the Metabolic Origins of the Sulfur and 3 -Nitrogen Atoms in Biotin of Escherichia coli by Mass Spectrometry. Biochemistry 23: 558-562, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00256-01 LB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enzyme Inactivation in Red Cells during Aging

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

PI:	Bong-whan Ahn	Visiting Fellow	LB, NHLBI
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB, NHLBI
	C. N. Oliver	Staff Fellow	LB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)
- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Over the past several years we have studied enzyme inactivation which is catalyzed by a variety of mixed function oxidation (MFO) systems. Many of the enzymes which we have found are inactivated by MFO systems in vitro have been found by others to accumulate in inactive or less active forms during aging. Levine has shown that E. coli glutamine synthetase and other key metabolic enzymes which are oxidatively modified in vitro by MFO systems generate a carbonyl protein derivative which becomes labeled when reduced with sodium borotritide or forms a stable hydrazone when treated with 2,4-dinitrophenylhydrazine (DNPH). We have used this property to develop reagents and presumptive screening assays for the presence of oxidatively modified proteins in cell extract preparations. We have chosen the human erythrocyte as a model for aging. It is well known the human erythrocytes are present in peripheral circulation for approximately 120 days and over that time period erythrocytes exhibit age related changes, such as a decrease in the specific activity of some enzymes. We have fractionated erythrocytes according to age (density) and we have found that the decrease in the specific activity of some enzymes is accompanied by an increase in the level of oxidatively modified protein.

Project Description:

Objectives: The primary objective of this project is to investigate the possible physiological role of oxidative modification in biological systems as a function of age. We have undertaken studies to determine whether enzymes which accumulate in human erythrocytes as inactive or less active forms are oxidatively modified and whether the modifications are similiar to the modifications generated by MFO systems *in vitro*.

Major Findings:

Inactivation of *E. coli* glutamine synthetase (GS) by MFO systems *in vitro* involves the oxidative modification of 1 of 16 histidine residues in each GS subunit with the generation of a carbonyl protein derivative. This derivative reacts with sodium borotritide or 2,4-dinitrophenylhydrazine (DNPH) to form a stable tritium labeled protein or stable hydrazone, respectively. We have used this property as presumptive evidence for the presence of oxidatively modified proteins in human erythrocytes fractionated by age using Percoll gradients. We have found that the specific activities of glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and aspartate aminotransferase decreases approximately 50% in old cells compared to young cells. Moreover, the level of oxidized protein was significantly greater in old cells compared to young cells.

Because even small amounts of residual hemoglobin in erythrocyte hemolysates interfere with spectral determination of DNPH-proteins and because high background labeling is observed in hemolysates treated with sodium borotritide even after acid hydrolysis, studies were undertaken to synthesize and characterize tritium labeled phenylhydrazine as a carbonyl reagent. The tritium labeled phenylhydrazine was prepared by an exchange reaction using tritiated H_2O and Adam's reagent. This new carbonyl reagent was found to be both highly sensitive and specific for carbonyl groups.

Proposed Course:

Our preliminary results have suggested that enzymes which accumulate in erythrocytes as inactive or less active forms during aging may be oxidatively modified. We are in the process of purifying several selected enzymes in order to generate polyclonal high affinity antibodies. Using these antibody preparations, we plan to isolate these enzymes from various erythrocyte fractions in order to measure directly the carbonyl content of each enzyme and to study the modifications which may occur *in vivo*.

Publications:

Oliver, C. N., Ahn, B., Wittenberger, M. E., and Stadtman, E. R.: Oxidative inactivation of enzymes: Implication in protein turnover and aging. In Ebashi, S. (Ed.): Cellular Regulation and Malignant Growth. New York, Academic Press, 1984, in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00257-01 LB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation and Characterization of Phosphoprotein Phosphatases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Emily Shacter	Staff Fellow	LB, NHLBI
Others:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB, NHLBI
	Joseph E. McClure	Medical Staff Fellow	CB, NHLBI
	Edward D. Korn	Chief, Laboratory of Cell Biology	CB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- xx☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Phosphoprotein phosphatases regulate cellular metabolism together with protein kinases by modulating the level of phosphorylation and, hence, specific activity of regulatory proteins. A variety of multimeric phosphatases have been isolated and grouped into several classes, termed type 1 (ATP-Mg-dependent) and types 2A (e.g., eIF-2 phosphatase and smooth muscle phosphatase I), 2B (calcineurin) and 2C; they differ in their sensitivity to inhibitor proteins and divalent cations and in their substrate specificity. However, definitive characterization of the enzymes has been hampered by difficulties in maintaining their native state throughout biochemical purification procedures. To elucidate the relationship between different phosphoprotein phosphatases, highly purified enzymes from soil amoebae, turkey gizzards, bovine heart and brain, and rabbit skeletal muscle and reticulocytes, were tested for antigenic relatedness. Two heterologous antibody preparations were employed for this purpose -- one was made against an Acanthamoeba type 2A phosphatase and the other was made to bovine brain phosphatase type 2B (gift of Claude Klee). Specific subunit cross-reactivity was examined by protein blot ("Western") analysis. In addition, conditions were sought for purification of the type-2A phosphatase by immunoaffinity techniques. To this end, a modification of the dot immunobinding assay was developed and employed to screen for reagents to dissociate the enzyme gently from the immune complex without destroying enzyme activity. Immunoaffinity columns were prepared and tested for their ability to remove the enzyme from solution. Finally, immunotitrations were performed to determine the specific antibody titer and test for inhibition of enzyme activity.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives:

(1) Study the role of phosphoprotein phosphatases in regulating cellular metabolism.

(2) Identify *in vivo* regulators for the type 2A phosphatases.

(3) Elucidate the relationship between different phosphoprotein phosphatases.

Major Findings: Antibodies (IgG) made in sheep to an Acanthamoeba phosphoprotein phosphatase (type 2A) were tested by protein blot analysis for cross-reactivity with pure phosphoprotein phosphatases from different tissues and species. The antibody reacted with every type 2 phosphatase tested, whereas it did not react with any type 1 phosphatase; thus, the catalytic subunits of turkey gizzard smooth muscle phosphatase-1 (type 2A), rabbit reticulocyte eIF-2 phosphatase (type 2A), bovine cardiac phosphatase (type 2A), and bovine brain calcineurin (type 2B) all cross-reacted, whereas the ATP-Mg-dependent phosphatase (type 1) and the type 1 catalytic subunit (35,000 M_r) did not. This degree of evolutionary conservation suggests a strong selective pressure on maintaining one or more features of these important regulatory enzymes. In addition, it may account for the difficulties encountered in making antibodies to the type 2 phosphatases and to the low titers obtained consistently by researchers in the field.

A solid phase assay was developed to serve as a rapid and easy method to screen for conditions under which specific antibody-antigen complexes can be dissociated, such as when performing immunoaffinity purification of immunoglobulins or proteins. In this procedure, the antigen (e.g., phosphatase) is absorbed onto nitrocellulose paper discs which are then blocked with 3% BSA in buffered saline and treated with primary antibody. Blots are then incubated with test elution solutions (e.g., 0.2 N acetic acid, pH 2.6, 15-30 minutes, room temperature) such as described for the multiple immunoreplica technique of Legocki and Verma (Anal. Biochem. 111, 385-392, 1981), or with control buffer. After washing with buffer and reblocking with BSA, the blots are incubated with a secondary antibody solution (e.g., peroxidase-conjugated or ^{125}I-labeled) and analyzed for binding (e.g., peroxidase staining or autoradiography). Comparison of the results obtained with the buffer control and those obtained with test elution solutions identifies clearly those solutions that remove the primary antibody and, hence, prevent binding of the secondary antibody. By this procedure, it was found that solutions of 2.5-4.0 $MgCl_2$ in BSA (or 10% glycerol) at mildly acidic pH (~ 5.0) dissociated completely the sheep antiphosphatase IgG from the bovine cardiac phosphatase at 4°C. Because Mg^{2+} protects the phosphatase from enzymatic decay and inhibition by various inhibitors (Shacter-Noiman, E. and Chock, P. B. (1983) J. Biol. Chem. 258, 4214-4219), these solutions are sufficiently mild to preserve phosphatase activity and may thus be employed for immunoaffinity purification of phosphoprotein phosphatases.

The sheep antibody did not inhibit the enzyme activity of the cardiac type 2A phosphatase at any concentration tested. Employing a solid phase assay for antibody-antigen dissociation described above, it was found that the apparent absence of enzyme inhibition by the antibody was not due to substrate-induced

dissociation of an immune complex. Moreover, all attempts failed to immunoprecipitate the phosphatase or to remove it from solution by affinity techniques. In the absence of any method to measure antibody-antigen binding in solution (i.e., not a solid phase), we cannot ascertain at this time whether the antibody recognizes the active sites of the enzymes or whether different domains were involved.

Both subunits of calcineurin (16,500 and 61,000 M_r) reacted with the sheep antibody. The antigenic relatedness of calcineurin and the bovine cardiac phosphatase (38,000 M_r) were analyzed further by testing for cross-reactivity with a polyclonal antibody made to highly purified calcineurin. Protein blot immunoanalysis showed that both the cardiac phosphatase 2A and brain calcineurin (both subunits) reacted with both antiphosphatase 2A and anticalcineurin antibodies. It should be pointed out, however, that the reaction of anticalcineurin with the type 2A enzyme was very faint. Nonetheless, the mutual cross-reactivity poses an intriguing problem because these enzymes appear to be so different; aside from the obvious molecular differences, calcineurin is regulated by Ca^{2+} calmodulin and by the 16,500 M_r calcium-binding subunit while no such regulatory subunit or mechanism has been identified for the type 2A enzymes. Moreover, the 38,000 M_r type 2A catalytic subunit, initially generated by ethanol treatment of crude extracts, has not been identified as such in vivo, thus leading to the hypothesis that it exists as part of a high molecular weight complex in the cell. The finding that phosphatases 2A and 2B are antigenically related raises the possibility that they are genetically related as well. But the immunological studies carried out so far do not reveal whether their polypeptide chains are related or whether the cross-reactivity results from the immunogenicity of common covalent modifying groups. For example, the NH_2-terminus of the 16,500 M_r subunit of calcineurin is myristylated; the NH_2-terminus of the 61,000 M_r subunit is also blocked, as are those of the type 1 and type 2A catalytic subunits, but the nature of these blocking groups has not yet been identified. Another protein which is myristylated is the catalytic subunit of cAMP-dependent protein kinase. This protein, too, showed faint cross-reactivity with both antiphosphatase 2A and anticalcineurin antibodies, thus supplying circumstantial evidence that myristic acid may be a common antigenic determinant. However, more direct studies will have to be carried out to define the antigenic domains.

Another interesting finding from the protein blot analyses was that although each of the multimeric phosphatases tested contained an ~ 60,000 M_r component, these polypeptides were antigenically distinct; that is, anticalcineurin did not react with the ~ 60,000 M_r component of the ATP-Mg-dependent phosphatase, and, while antiphosphatase 2A reacted with the 61,000 M_r subunit of calcineurin, it did not bind to the ~ 60,000 M_r components of either eIF-2 phosphatase or SMP-I. Although the ~ 60,000 M_r components that copurify with the type 1 and type 2A phosphatases are referred to as subunits, their role in regulating the associated enzymes has not been shown and cannot be assumed.

Proposed Course: Define the antigenic domain(s) that are common to the type 2 phosphatases and absent from the type-1 phosphatases. Study the relationship between phosphatase 2A and calcineurin.

(a) Do peptide maps and immunoanalyses on cardiac phosphatase 2A and calcineurin.

(b) Identify enzymatically and chemically any covalent modifying groups that may confer antigenic cross-reactivity to the enzymes.

(c) Isolate native phosphatase 2A from bovine cardiac muscle and carry out immunological and structural comparisons with calcineurin.

Publications:

Shacter-Noiman, E., Chock, P.B., and Stadtman, E.R.: Protein phosphorylation as a regulatory device. Phil. Trans. R. Soc. Lond. B. 302: 157-166, 1983.

Jurgensen, S., Shacter, E., Huang, C.Y., Chock, P.B., Yang, S.-D., Vandenheede, J.R., and Merlevede, W.: On the mechanism of activation of the ATP·Mg(II)-dependent phosphoprotein phosphatase by kinase F_A. J. Biol. Chem. 259: 5864-5870, 1984.

Shacter, E.: Organic extraction of P_i with isobutanol/toluene. Anal. Biochem. 138: 416-420, 1984.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1983 through September 30, 1984

The experimental interests of the Cardiology Branch focus on 1) elucidating the mechanisms responsible for dynamic alterations in coronary vascular resistance; 2) defining the pathophysiology and treatment of coronary artery disease, angina pectoris, and hypertrophic cardiomyopathy; 3) evaluating and treating valvular heart disease; and 4) exploring the etiologies and treatment of dilated cardiomyopathy.

DYNAMIC CORONARY VASOCONSTRICTION AS A CAUSE OF MYOCARDIAL ISCHEMIA

Traditionally, angina pectoris has been considered to result from a fixed stenosis limiting blood flow to the myocardium. As myocardial oxygen demands increase in response to stress, the stenosis restricts the increase in flow that normally occurs, causing myocardial oxygen demands to exceed myocardial oxygen supply: ischemia, and ultimately anginal pain result. The recognition of vasospastic angina (Prinzmetal, or variant angina) focused attention on the fact that intrinsic vasoconstrictor tone of coronary vessels can alter sufficiently to cause myocardial ischemia. However, the concept of vasospastic angina was limited, being conventionally understood as angina precipitated at rest by a profound decrease in coronary flow due to spasm-induced total or near-total occlusion of a large epicardial coronary artery. Over the past two years we have explored the possibility that dynamic coronary vasoconstriction may not only involve large epicardial coronary vessels, but also the small coronary arteries or coronary arterioles, which are responsible for autoregulatory changes in coronary vascular resistance.

Ischemia caused by small coronary artery vasoconstriction in pts with atypical angina: We have this year extended our study of the mechanisms responsible for angina pectoris occurring in pts with variable threshold angina but normal coronary arteries. We have now identified 50 such pts who underwent measurements of great cardiac vein flow (draining the LAD coronary artery) at rest and with atrial pacing to achieve heart rates of 150. Pacing ordinarily increases coronary flow and decreases coronary vascular resistance. Patients experiencing angina during pacing either under control conditions or during administration of vasoconstrictor stimuli (ergonovine infusion) increased LAD flow and decreased LAD resistance less than pts who did not experience angina. We have now further documented that the chest pain and abnormal flow patterns manifest by these pts are associated with myocardial ischemia: pts with abnormal vasodilator reserve exhibited diminished myocardial lactate consumption, a greater arterial-venous oxygen difference across the myocardium, and a marked increase in LVEDP from rest to pacing. We then hypothesized that if ischemia were indeed occurring, we should be able to detect LV dysfunction. Hence, these pts underwent rest and exercise radionuclide angiography. During supine bicycle ergometry over two thirds of the pts with abnormal vasodilator reserve manifest abnormalities in LV contraction consisting either of a decrease in ejection fraction during exercise, or the appearance of regional wall motion abnormalities. Quantitative coronary angiography demonstrated no significant

lumenal narrowing of the epicardial coronary arteries in response to ergonovine. Thus, these data are consistent with the hypothesis that some pts with chest pain and angiographically normal-appearing coronary arteries have dynamic abnormalities of the small coronary arteries or coronary microcirculation that cause abnormal vasodilator reserve or coronary constriction. These abnormalities result in myocardial ischemia which, in turn, causes angina pectoris, hemodynamic abnormalities, and impairment of LV systolic and diastolic function.

Pharmacologic management of pts with abnormal coronary vasodilator reserve: These pts exhibit abnormal vasoconstrictor tone influencing the small coronary arteries, and because calcium antagonists exert their major vasodilator effect at the arteriolar level, we determined whether calcium antagonists could control the frequency of anginal symptoms. Pts were given either verapamil or nifedipine in an outpatient study that was randomized and double-blinded. The number of anginal attacks and frequency of nitroglycerin consumption were markedly reduced by the calcium antagonists.

Small coronary artery constriction and myocardial ischemia caused by endogenously present neuropeptides and vasoactive amines: To determine whether myocardial ischemia could be caused by endogenous substances known to be present in the coronary vasculature or present in the blood, several animal studies were conducted in dogs to determine whether neuropeptide tyrosine, (a peptide present in high concentrations in the coronary vessels), and vasopressin, (present in high concentrations in the blood following stress) could produce ischemia when infused into the coronary artery of dogs. Intramyocardial pH changes and determinations of left ventricular function were used as indices for the presence or absence of myocardial ischemia. Both agents caused dose related decreases in pH and LV function. Moreover, differential pressure measurements of the large and small coronary vessels demonstrated that the vasoconstriction leading to ischemia occurred at the small vessel level. Thus, endogenous vasoactive substances can cause severe small vessel coronary vasoconstriction such that the resulting increases in coronary vascular resistance can be sufficiently profound so as to override autoregulatory influences, causing both ischemia and myocardial dysfunction.

Influence of alterations in blood lipids and coronary atherosclerosis on coronary vascular reactivity: In this study we examined the question of whether an atherosclerotic diet administered to pigs, resulting in elevated LDL cholesterol and, subsequently, in atherosclerosis, alters vascular responsiveness to vasoconstrictor agents. Pigs were fed an atherosclerotic diet and studied 1) in several weeks when serum LDL cholesterol levels were elevated, but atherosclerosis had not occurred, and 2) in several months when atherosclerotic lesions were present.

Our preliminary studies suggest that vascular reactivity is altered by the elevation in LDL cholesterol as well as by the development of atherosclerosis.

Thrombosis as a contributing cause of coronary obstruction in pts with chronic refractory angina and with unstable angina: It has been amply demonstrated that acute development of thrombus is an important precipitating cause of acute myocardial infarction (AMI) in a substantial percentage of patients with AMI. It also has been hypothesized that thrombus formation is etiologically important in the development of atherosclerosis. This concept postulates that thrombus forms on the endothelium of the vessel wall, organizes and then retracts; repetitions of these events lead in a stuttering course to the development of significant coronary obstruction and to angina, and when the thrombus totally occludes the artery, to AMI. To determine the potential contribution of coronary thrombus to severe chronic stable angina, thrombolytic therapy using ancrod, a snake venom derivative with defibrinogenating and indirect tissue plasminogen activator properties, was administered to 10 pts, all of whom had chronic incapacitating angina refractive to traditional therapy. Pts were randomized and treated blindly with daily infusions of either placebo or ancrod for two weeks. Following ancrod therapy, all pts increased exercise duration greater than 50% and 3 pts with severe rest angina became symptom free. However, improved exercise duration was not associated with improved ST segment changes, peak pressure rate product during exercise, or reduced radionuclide derived indices of ischemia. Moreover, after ancrod therapy, no instances of recannalization were observed in native coronary vessels or bypass grafts, as assessed by coronary angiography. Thus, thrombolytic therapy using ancrod in refractory angina improves exercise capacity and symptoms in most pts, but the mechanism underlying beneficial responses is undefined. Moreover, although many pts continued to exhibit improved symptoms for weeks or months following therapy, ultimately most experienced a recurrence of their previous incapacitating symptoms. Hence, thrombus formation and thrombolytic therapy does not appear to hold great promise in the treatment of pts who have already developed severe symptomatic coronary disease.

Studies will be initiated shortly to determine the role of thrombus and thrombolytic therapy in the pathogenesis and treatment of pts with unstable anginal syndromes.

Percutaneous transluminal coronary angioplasty (PTCA) in the treatment of coronary artery disease: Balloon angioplasty, or PTCA, successfully reduces the degree of coronary obstructions and improves both symptoms and exercise capacity. To evaluate the incidence and causes of coronary restenosis after successful PTCA, 251 consecutive patients treated either at the NIH or at Georgetown University Medical Center were evaluated. In the initial group of 89 successfully treated pts re-studied 1-11 months after PTCA, restenosis occurred in 36%. Restenosis rate was only 17% in the last 100 pts who initially had successful PTCA. This improvement was probably related to higher inflation pressures that could be achieved with newer balloons. Overall mortality for the procedure was 0.08%. With repeat PTCA of pts with restenosis, success rate was over 80%.

Although the short-term beneficial effects of PTCA have been unequivocally documented, the long-term efficacy of this procedure is undefined. We therefore re-evaluted 28 of the first 30 patients who had successful PTCA without clinical or anatomic evidence of restenosis in the first 9 months. Re-evaluation was carried out an average of 3 years (28-46 months) after successful PTCA. At catheterization percent coronary diameter narrowing pre PTCA was 67%, improving to 31% immediately after the procedure. It was 28% six months later and 20% at late follow up, a value significantly less than the narrowing found at the six month study. Treadmill exercise time was 7.5 minutes pre PTCA. and increased $2^1/2$ times at all post PTCA studies (within one week, 6 months and 3 years later). The change in ejection fraction from rest to exercise was also improved by PTCA shortly after the procedure, an effect maintained over the 3 years. Most pts exhibited markedly improved symptoms maintained over the 3 year follow up period. Of note, 2 pts who developed recurrent symptoms late post PTCA demonstrated persistent improvement in the previously dilated vessel but had developed new stenoses at nondilated sites. Thus, the short-term anatomic and functional success after PTCA is maintained for at least 3 years, even though disease occasionally progresses at other sites.

HYPERTROPHIC CARDIOMYOPATHY

Does true "obstruction" to LV outflow exist in patients with HCM?: A major controversy persists as to whether true outflow obstruction exists in pts with HCM and subaortic gradients. This question is of critical importance in terms of understanding the basic pathophysiologic mechanisms present in this disease, as well as the potential role of operation, since the operation is designed only to eliminate or reduce the degree of obstruction. We therefore analyzed with pulsed Doppler echocardiography the patterns of LV emptying in 50 pts with HCM (20 with and 30 without gradients) and in 20 normals. The argument offered by investigators claiming that no obstruction exists is based on the perception that a gradient is recorded only after the LV emptying is completed; hence, true obstruction causing an impediment to LV outflow could not exist. Our study demonstrated that over 40% of forward flow occurred after mitral-septal contact (an event marking onset of gradient and elevated ventricular pressures). Mid-systolic impedance to outflow was also suggested by the rapid deceleration in aortic flow velocity concomittant with mitral-septal contact, and premature partial aortic valve closure. Furthermore, LV ejection was prolonged and the LV continued to empty and shorten during the period when both pressure gradient and high LV pressures were present. In contrast, pts without obstructive HCM exhibited no evidence of impeded LV ejection. In another study HCM pts were evaluated at catheterization in which LV pressure volume loops were determined. We found that over 60% of LV stroke volume was ejected after onset of the outflow tract gradient appeared. These results demonstrate that in HCM: 1) SAM produces a mechanical obstruction to LV emptying and a considerable portion of the stroke volume is impeded in its egress from the LV; 2) the gradients are of pathophysiologic importance since the LV continues to contract in the presence of markedly elevated ventricular pressures.

Mechanisms contributing to ischemia in HCM: Chest pain is a frequent symptom of pts with HCM, despite the absence of large vessel coronary disease. HCM pts often experience pain with variable threshold onset, including rest pain. The pain is often prolonged even after cessation of activity. To determine

the mechanisms responsible for ischemia, 20 pts with HCM and chest pain, despite angiographically normal coronary arteries, underwent a pacing study with measurement of great cardiac vein flow. The results were compared to those of 28 pts without HCM who, after evaluation, were thought to be normal. Flow increased linearly in the control group up to a HR of 150. While flow in HCM pts initially rose when paced to intermediate HR (120), 12 of 20 pts experienced their typical chest pain. With continued pacing to HR 150, flow actually fell in 14 HCM pts, with 18 of 20 experiencing chest pain and metabolic evidence of myocardial ischemia. LVEDP rose substantially, concomitant with the fall in flow. Thus, most HCM pts achieve maximal coronary vasodilatation at modest increases in HR. Myocardial ischemia occurs in many pts at this point, resulting in elevation in LVEDP (probably because of ischemia-related changes in ventricular compliance), with a subsequent adverse effect on coronary flow. Such mechanisms could lead to persistent ischemia even after elimination of the ischemia-precipitating stimulus -- once ischemia occurred, the resulting increased LV filling pressures would further compromise myocardial flow and lead to an independent self-sustaining mechanism causing persistent ischemia. These results also suggest that agents tending to lower LV filling pressures or improve compliance (such as diuretics and verapamil), may be beneficial in alleviating ischemia-related symptoms.

An additional study was carried out to determine whether such HCM pts are abnormally sensitive to vasoconstrictor stimuli. We hypothesized that if maximal vasodilation occurred with relatively low myocardial metabolic stress, vasodilator reserve would have been exhausted, thereby making it difficult for the coronary system to accommodate to vasoconstrictor stimuli. We therefore studied great cardiac vein flow at rest and during pacing before and after ergonovine (.15 mg iv). Those HCM pts who achieved maximal vasodilator reserve at intermediate paced HR demonstrated a decrease in peak flow with ergonovine. This was not observed in those HCM pts who exhibited relatively normal increases in flow. This sensitivity to a vasoconstrictor stimulus may explain the variation in anginal threshold many HCM pts experience.

Regional LV asynchrony and impaired global LV diastolic filling in HCM: LV diastolic filling is impaired in HCM. To assess the influence of LV regional synchrony on global filling, we studied 48 HCM pts by radionuclide angiography before and during oral verapamil therapy. We found that in many HCM pts, impaired global LV filling results, at least in part, from nonuniformity of systolic function. Some regions of the LV completed shortening early and some late. The delayed systolic shortening probably contributes to impaired global LV relaxation, thereby interfering with LV filling. Verapamil administration over 1-2 weeks improved diastolic function, a change associated with a reduction in the asynchrony of LV contraction.

To determine the mechanism responsible for the beneficial symptomatic effects of verapamil in pts with HCM, we compared the relation between changes in LV filling and changes in exercise capacity after verapamil. Exercise capacity increased in 28 of 32 pts manifesting an improvement in diastolic function, but in only 3 of 11 pts with unchanged diastolic function. This initial trend persisted in 18 pts studied after 1 year of

therapy: 10 of 11 pts with a persistent improvement in diastolic function had persistent improvement in exercise tolerance, compared to only 1 of 7 pts in whom diastolic funtion was unchanged or decreased relative to pre-verapamil levels. The short and long-term changes in diastolic function also correlated with changes in functional class. These data indicate that enhanced LV diastolic function is an important mechanism contributing to the clinical improvement experienced by many HCM pts during verapamil therapy.

Amiodarone in pts with HCM and refractory symptoms: An increasing subgroup of pts with HCM and refractory cardiac symptoms have provided the impetus for investigating new forms of drug therapy. Amiodarone is a drug with potent antiarrhythmic effects. Because one of our HCM pts treated with this drug for arrhythmias also demonstrated marked symptomatic improvement unrelated to antiarrhythmic effects, we studied the efficacy of this drug in 10 HCM pts with refractory symptoms. Exercise duration increased more than 100% with amiodarone in 7 of 10 pts. Ventricular tachycardia during ambulatory monitoring was present in 6 pts on no medications, or during best medical therapy, but was absent with amiodarone. To determine the mechanisms whereby amiodarone improves cardiac symptoms, several analyses were performed. Improved exercise capacity did not correlate with amiodarone induced changes in heart rate, other resting or exercise hemodynamic parameters, QT interval, or amiodarone blood levels. Amiodarone also did not alter indices of LV systolic or diastolic function. Thus, amiodarone significantly improves exercise capacity and symptoms in many pts with HCM and refractory symptoms, and may be used when other approaches to symptomatic control fail. However, its mechanism of action and long-term efficacy are unknown.

Progression of LV hypertrophy during adolescence in HCM: HCM has been considered to be a congenital cardiac malformation with LV hypertrophy often evident shortly after birth. To determine whether LV mass is altered later in life, 21 asymptomatic and untreated relatives of pts with HCM were studied serially with echocardiography, initially at age 8-15 yrs (mean 11) and most recently at 13-19 yrs (mean 15). Over a 3-5 year follow-up 11 pts showed a marked increase in the magnitude and distribution of LV hypertrophy, including 3 pts with progression from a normal ventricle. Thus, the morphologic expression of HCM can spontaneously appear or progress substantially in genetically predisposed individuals during adolescence.

VALVULAR HEART DISEASE

Influence of preoperative LV function on survival after AV replacement for AR: Recent studies suggest that LV function may no longer be an important determinant of survival after operation (AVR) for aortic regurgitation (AR) because of improved operative techniques. To assess the effect of LV function on prognosis in the current surgical era, we performed echocardiography and radionuclide angiography on 94 consecutive pts undergoing AVR from 1976 to 1983. Cold hyperkalemic cardioplegia (HKCP) was used for preservation in 49 pts. Preoperatively LVEF and fractional shortening were the most significant predictors of survival: 5 year survival was 71% in pts with subnormal EF compared to 95% with normal EF. Survival was not influenced by HKCP and HKCP did not alter the influence of LV function on prognosis. Hence, despite the improved operative techniques and better long-term survival compared to earlier results, preoperative resting

LV dysfunction continues to identify pts with AR at risk of death after AVR. Early operation in such pts may result in further improvement in survival.

The natural history of mitral regurgitation (MR): Previous studies have demonstrated that LVEF of pts with MR deteriorates significantly following valve replacement. However, the long-term clinical significance and effect on long-term LV function of this finding are unknown. We therefore studied the effects of mitral valve replacement on early post-operative (6 months to 2 years) and late post-op (3-5 years) LV function in 23 MR patients. Before MVR, EF was 48%; EF early after MVR decreased to 35%. The only pt who died during subsequent follow-up was the pt with the lowest ejection fraction early post-op (13% compared to 35% pre-op). All remaining pts are alive with 19 of 22 having persistent improvement in functional class. At 3-5 years EF was 39%, a significant increase from the early post-op studies. All pts with EF greater than 30 on early study remain stable or showed an increase in EF on late study. However, of 8 pts with EF less than 30% on early study, one died, 2 had no change in EF between early and late studies, and 3 had a progressive decrease in EF. Hence, early post-op deterioration in EF does not generally correlate with subsequent clinical or LV functional course; most pts with substantial early decrease in EF manifest improved long-term LV function with good clinical results 3-5 years post-op. However, pts with profound decreased EF post-op (less than 30%) appear to constitute a group at risk of persistent LV dysfunction.

DILATED CARDIOMYOPATHIES

The Cardiology Branch is collaborating with the Medical Intensive Care Unit in a study designed to define the natural history of dilated cardiomyopathy (DCM) and the effects of anti-inflammatory treatment. In this randomized study the effects of standard medical treatment will be compared with those of standard medical treatment plus anti-inflammatory therapy (prednisone and, if not effective, cytoxan). End points are changes in functional status, myocardial histology and function, and survival. To date, over 50 pts have been screened and 30 pts entered into the study. It is hoped that we eventually will be able to identify pts at high risk exhibiting progressive LV dysfunction, of defining underlying causes of the DCM, and of determining whether or not anti-inflammatory therapy is effective therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-01670-08 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ventricular ectopy in asymptomatic and symptomatic patients with AR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Ulla Idanpaan-Heikkila, M.D.	Visiting Scientist	CB NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Prognosis in patients with aortic regurgitation is usually determined by the status of left ventricular (LV) function. However, when death occurs, it is often sudden and may be arrhythmogenic in origin. The present study utilized 24-hour ambulatory monitoring to evaluate 133 consecutive patients with isolated aortic regurgitation for the occurrence of ventricular arrhythmias. The frequency and severity of the ventricular arrhythmias that were detected correlated with measurements of LV dimensions or function, and exercise capacity. Asymptomatic patients had a lower frequency of ventricular ectopic beats and less high grade ventricular events (two or more in a row) compared to symptomatic patients before operation and patients who had had their aortic valves replaced. There was no difference in the frequency or severity of arrhythmias between the latter two groups. Those patients in the symptomatic and operated groups who had high grade and increased frequency of arrhythmias had depressed rest and exercise LV function compared to patients in those groups without high grade arrhythmias. In addition, patients with high frequency and grade arrhythmias also had larger LV dimensions. Exercise capacity was similar in patients with low and high grade arrhythmias. During a mean follow-up time just over three years, there have been 10 deaths in these patients, but only two of them could be attributed to ventricular arrhythmias.

This work demonstrates that increased frequent and grade of ventricular ectopy is common in patients with aortic regurgitation and appears to be related to LV dysfunction. The ventricular ectopy did not appear to be related to the occurrence of sudden death, although chronic antiarrhythmic therapy may have had a protective effect and contributed to the low mortality. Also follow-up time was relatively short.

GPO 904-917

Project Description:

Prognosis in patients with aortic regurgitation is usually determined by the status of left ventricular (LV) function. However, when death occurs, it is often sudden and may be arrhythmogenic in origin. The present study utilized 24-hour ambulatory monitoring to evaluate 133 consecutive patients with isolated aortic regurgitation for the occurrence of ventricular arrhythmias. Patients also underwent echocardiography, graded treadmill exercise testing, and rest and exercise radionuclide angiography to see if the frequency and severity of the ventricular arrhythmias that were detected correlated with measurements of LV dimensions or function, and exercise capacity. Asymptomatic patients had a lower frequency of ventricular ectopic beats and less high grade ventricular events (two or more in a row) compared to symptomatic patients before operation and patients who had had their aortic valves replaced. There was no difference in the frequency or severity of arrhythmias between the latter two groups. Those patients in the symptomatic and operated groups who had high grade and increased frequency of arrhythmias had depressed rest and exercise LV function compared to patients in those groups without high grade arrhythmias. In addition, patients with high frequency and grade arrhythmias also had larger LV dimensions. Exercise capacity was similar in patients with low and high grade arrhythmias. During a mean follow-up time just over three years, there have been 10 deaths in these patients, but only two of them could be attributed to ventricular arrhythmias.

This work demonstrates that increased frequent and grade of ventricular ectopy is common in patients with aortic regurgitation and appears to be related to LV dysfunction. The ventricular ectopy did not appear to be related to the occurrence of sudden death, although chronic antiarrhythmic therapy may have had a protective effect and contributed to the low mortality. Also follow-up time was relatively short.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-01672-08 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Maintenance of a computerized clinical data bank for cardiology patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Charles McIntosh, M.D.	Senior Surgeon	SU NHLBI
J. Emmett Ward	Chief, DMB	DMB DCRT
Gail Greenberg	Computer Technician	CB NHLBI
Lavonne Dragt	Computer Technician	CB NHLBI
Richard Clark, M.D.	Chief, Heart Surgery Br	SU NHLBI
Larry Martin	Systems Analyst	DMB DCRT
Michael Jones	Senior Surgeon	SU NHLBI

COOPERATING UNITS *(if any)*

Data Management Branch (DMB), DCRT
Surgery Branch, NHLBI

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	.1	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits, as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description; x-ray; ECG; catheterization; radionuclide; exercise stress test; ambulatory monitor and echocardiogram results; listing of current medications; and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978, and current and retroactive information has been entered on all of our patients. The data base of the Cardiology Branch and that of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 20 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Cardiology and Surgery Clinics in order to make chart reviews easier at these visits.

At the present time, there are 7,688 patients entered into the data base. Of these patients, approximately 4,200 have undergone some cardiac operation.

Project Description:

A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits, as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description; x-ray; ECG; catheterization; radionuclide; exercise stress test; ambulatory monitor and echocardiogram results; listing of current medications; and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978, and current and retroactive information has been entered on all of our patients. The data base of the Cardiology Branch and that of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 20 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Cardiology and Surgery Clinics in order to make chart reviews easier at these visits.

At the present time, there are 7,688 patients entered into the data base. Of these patients, approximately 4,200 have undergone some cardiac operation.

The present system is being totally reevaluated in order to make the data contained more "user friendly"; i.e., to make accessibility and manipulation of data more practical. Several site visits have occurred and restructuring of the data base is being planned.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-HL-01750-06 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Verapamil: A new approach to the treatment of hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Barry J. Maron, M.D.	Head, Echo Lab	CB	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have demonstrated that verapamil reduced left ventricular outflow tract obstruction and improved exercise capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in hypertrophic cardiomyopathy, 227 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital. Fifty-nine percent of patients discharged on the drug have remained on verapamil for periods as long as 53 months. The major reason the drug was stopped in patients discharged on verapamil was because symptoms were unrelieved or recurred. Less than 2% of patients discharged on verapamil had the drug discontinued because of non-physiologic drug side effects. Nine patients have died while on chronic drug treatment. Eight of these deaths were related to cardiovascular events but whether they occurred because of or despite verapamil therapy is uncertain. Adverse hemodynamic effects experienced by the patients include 17 episodes of pulmonary congestion, 12 of hypotension, 5 cases of sinus arrest, approximately 13% incidence of development of junctional rhythm or Wenkebach second degree heart block. In almost all cases of hypotension, junctional rhythm, second degree heart block, and non-cardiovascular problems, the drug was continued at reduced dosage, although therapeutic efficacy was sometimes compromised because of an inability to use higher doses.

We have been able to identify those subgroups of patients who appear to be at increased risk for developing complications secondary to verapamil administration, and who should not receive the medication. In addition, it appears that its primary mechanism of therapeutic action may be due to its inability to improve early filling of the ventricle.

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Project Description:

The primary medical approach to symptomatic therapy in patients with hypertrophic cardiomyopathy (HCM) was formerly the use of beta blocking agents. We have demonstrated that verapamil reduced left ventricular outflow tract obstruction and improved exercise capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in hypertrophic cardiomyopathy, 227 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital between September 1977 and June 1982. Fifty-nine percent of patients discharged on the drug have remained on verapamil for periods as long as 53 months. The major reason the drug was stopped in patients discharged on verapamil was because symptoms were unrelieved or recurred. Less than 2% of patients discharged on verapamil had the drug discontinued because of non-physiologic drug side effects. Nine patients have died while on chronic drug treatment. Eight of these deaths were related to cardiovascular events (one was the result of a gastrointestinal malignancy), but whether they occurred because of or despite verapamil therapy is uncertain. Adverse hemodynamic effects experienced by the patients include 17 episodes of pulmonary congestion, 12 of hypotension, 5 cases of sinus arrest, approximately 13% incidence of development of junctional rhythm or Wenkebach second degree heart block. In almost all cases of hypotension, junctional rhythm, second degree heart block, and non-cardiovascular problems, the drug was continued at reduced dosage, although therapeutic efficacy was sometimes compromised because of an inability to use higher doses.

Due to the ongoing work in this area, we have been able to identify those subgroups of patients who appear to be at increased risk for developing complications secondary to verapamil administration, and who should not receive the medication. In addition, it appears that its primary mechanism of therapeutic action may be due to its inability to improve early filling of the ventricle.

Publications:

Rosing, D.R., Idanpaan-Heikkila, U., Maron, B.J., Bonow, R.O., Epstein, S.E. The use of calcium channel blocking drugs in the treatment of patients with hypertrophic cardiomyopathy. Am J Cardiology. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-HL-01761-06 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Three year follow-up of patients undergoing PTCA

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) Name (Last, first, middle initial)

Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Richard O. Cannon, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB	NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Rita Mincemoyer	Cath Lab Nurse	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although it has been observed that patients describe marked symptomatic benefit and improved exercise capacity 2-4 years after the performance of percutaneous transluminal coronary angioplasty (PTCA), little data is available documenting anatomic and functional results during long-term follow-up. Thus, 28 of the first 30 patients who had a successful procedure, and had not had clinical or anatomical evidence of restenosis in the first 9 months after the procedure, were reevaluated at an average of 37 months after their last successful procedure. At follow-up patients underwent cardiac catheterization, treadmill exercise testing and radionuclide angiography at rest and with exercise.

The percent diameter narrowings pre-PTCA was 67±14% and improved to 31%±14% immediately after the procedure. It was 28±14% six months later and 20±14% at late follow-up. The last measurement is statistically significantly less than the narrowing found at the 6 month study. Treadmill exercise time was 7.5±7 minutes pre-PTCA and improved to 17.2±7, 19.8±7, and 17.5±7 minutes at the three respective post-PTCA studies. No change occurred in rest LV ejection fraction (EF) after PTCA, but exercise LVEF, which had fallen by 4.3±7.5% pre-PTCA (compared to rest), rose after PTCA by 8.2±7.5, 4.7±7.5, and 4.6±17.5% respectively. Fifteen patients showed a greater than 10% decrease in the amount of narrowing at the angioplasty site between the six month and three year studies. Two patients developed new significant stenoses at nondilated sites during follow-up. These results indicate that the short-term anatomic and functional success after PTCA is maintained for at least three years, even though disease occasionally progresses at other sites.

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Project Description:

Although it has been observed that patients describe marked symptomatic benefit and improved exercise capacity 2-4 years after the performance of percutaneous transluminal coronary angioplasty (PTCA), little data is available documenting anatomic and functional results during long-term follow-up. Thus, 28 of the first 30 patients who had a successful procedure, and had not had clinical or anatomical evidence of restenosis in the first 9 months after the procedure, were reevaluated at an average of 37 (range=27.5-45.5) months after their last successful procedure. Twenty-six patients had single vessel disease and two had multiple vessel disease. At follow-up patients underwent cardiac catheterization, treadmill exercise testing and radionuclide angiography at rest and with exercise.

The percent diameter narrowings pre-PTCA was 67±14% and improved to 31%±14% immediately after the procedure. It was 28±14% six months later and 20±14% at late follow-up. The last measurement is statistically significantly less than the narrowing found at the 6 month study. Treadmill exercise time was 7.5±7 minutes pre-PTCA and improved to 17.2±7, 19.8±7, and 17.5±7 minutes at the three respective post-PTCA studies. These three values are statistically longer than pre-PTCA but not different from each other. No change occurred in rest LV ejection fraction (EF) after PTCA, but exercise LVEF, which had fallen by 4.3±7.5% pre-PTCA (relative to the value at rest), rose after PTCA by 8.2±7.5, 4.7±7.5, and 4.6±17.5% respectively. The post-PTCA values are all statistically similar and significantly different from the pre-PTCA testing. According to the Canadian Heart Association functional classification, before PTCA 1 patient was asymptomatic, eight were functional class (FC) II, 16=FC III and 3=FC IV. At long-term follow-up 17 patients were asymptomatic, 4=FC I, 4=FC II, and 3 were still FC III. Fifteen patients showed a greater than 10% decrease in the amount of narrowing at the angioplasty site between the six month and three year studies. Two patients developed new significant stenoses at nondilated sites during follow-up and are two of the three patients who are still FC III. The third is a patient with multiple vessel disease. These results indicate that the short-term anatomic and functional success after PTCA is maintained for at least three years, even though disease occasionally progresses at other sites.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-HL-04015-03 CB

October 1, 1983 - September 30, 1984

Early degradation of collagen after acute myocardial infarction in the rat

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Jagdish W. Butaney, M.D.	Research Associate	PB NHLBI
Bruce McManus, M.D.	Research Associate	PB NHLBI
Edith Spier	Research Associate	CB NHLBI
Alan B. Krantz	Medical Student	Univ of Md
Roberto Bolli, M.D.	Div of Cardiology	Baylor Univ
Victor J. Ferrans, M.D., Ph.D.	Chief, Ultrastructure Sec.	PA NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI/IR

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cardiac rupture and aneurysm formation following acute myocardial infarction may be related to degradation of structurally important collagen molecules by inflammatory cell proteolytic enzymes. Using a rat model of acute myocardial infarction, significant collagen degradation was found to occur in the final 24 hours following experimental infarction. This degradation was associated with a marked increase in proteolysis in the infarct region. This collagen degradation was inhibited by eliminating inflammatory cells by prior whole body irradiation. Electron microscopic studies revealed greater preservation of collagen in the 29-hour old infarction of leukopenic rats than in those of controls. These results suggest that there is early collagen degradation after myocardial infarction which is mediated by inflammatory cells and may be of pathogenic importance in the development of cardiac rupture and aneurysm formation.

Project Description:

After acute myocardial infarction, proteolysis of necrotic myocardium is mediated by infiltrating inflammatory cells at the infarct margin. Collagen forms a structural fibroskeleton in healthy myocardium, and after acute myocardial infarction this collagen may continue to provide significant tensile strength to the necrotic muscle wall. To determine whether collagen is also degraded (which might decrease wall strength) and, if so, whether inflammatory cell protease are implicated, hydroxyproline was measured from infarct zone and normal zone tissue from 24-hour infarcts produced in control rats and rats made leukopenic (WBC $<300/mm^3$) by prior whole body irradiation. Hydroxyproline was measured after precipitation of tissue homogenate with trichlorocecetic acid to separate partially degraded collagen from larger collagen molecule that might retain structural importance.

Data reported as mean ± SD; nmol/mg dry wt. tissue.

	Unoperated Control	Control	Leukopenic
	n=20	n=19	n=20
NZ	10.6±0.8	10.6±1.1	11.5±1.5
IZ		6.9±0.7*	9.9±1.2

*=$p<0.01$
NZ=normal zone(septum); IZ=ischemic zone (free wall of left ventricle)

At 24 hours there was significant (25%) collagen degradation in the infarct zone in control rats but not in leukopenic rats. Tissue cell counts revealed a paucity of inflammatory cells in the infarct margin in leukopenic rats. Electron microscopic studies revealed greater preservation of collagen in the 24 hour old infarcts of irradiated leukopenic rats compared to those of control rats.

These results suggest that at 24 hours after experimental myocardial infarction, there is significant collagen degradation mediated by inflammatory cell protease.

Publication:

Cannon R.O., Butaney, J.W., McManus, B.M., Speir, E., Krantz, A.B., Bolli,R., Ferrans, V.J.: Early degradation of collagen after acute myocardial infarction in the rat. Am J Cardiol 52:390, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04022-04 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Relationship of blood nifedipine levels to hemodynamic effects in patients with HCM

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation.)

Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Lawrence Lesko	Dept of Pharmacology	Univ of Md
Sandro Betocchi, M.D.	Visiting Scientist	CB NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB NHLBI
Harold Ostrow	Elect Engineer	DCRT
Richard O. Cannon, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Department of Pharmacology, University of Maryland
Division of Computer Research and Technology

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.4	0.2

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although verapamil, the prototype calcium channel blocking agent, has been shown to have hemodynamic effects which should be of benefit to patients with hypertrophic cardiomyopathy, our studies with nifedipine, another calcium channel blocking drug, have not shown consistent beneficial effects. A gas chromatographic assay was used to measure blood nifedipine levels, which were then correlated with the hemodynamic effects.

Nifedipine levels plateaued 20 minutes after the administration of either 10 or 20 mg buccally and did not significantly increase even with the administration of a second 10 mg dose. The values were similar, independent of whether 10 or 20 mg was administered as the first dose. As expected, there was a linear relationship between nifedipine concentrations and increases in heart rate and decreases in peripheral vascular resistance. Cardiac output increased only as a result of heart rate changes. Pulmonary wedge pressure was also linearly related to nifedipine levels.

In contrast to our results with verapamil administration, left ventricular outflow tract obstruction was not affected at lower nifedipine levels (<80ng/ml), but actually increased (14±26%, $p<0.05$) at levels above this concentration. Also different from the verapamil findings was the fact that nifedipine had no effect on indicators of systolic and diastolic left ventricular function.

Thus we were not able to demonstrate any beneficial hemodynamic effects of buccally administered nifedipine to patients with hypertrophic cardiomyopathy. Moreover, at higher concentrations of the drug, there was a tendency for detrimental effects to occur, a reduction in stroke volume, an increase in left ventricular outflow tract obstruction, and an elevation of left ventricular filling pressures.

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Project Description:

Although verapamil, the prototype calcium channel blocking agent, has been shown to have hemodynamic effects which should be of benefit to patients with hypertrophic cardiomyopathy, our studies with nifedipine, another calcium channel blocking drug, have not shown consistent beneficial effects. In order to evaluate further the hemodynamic responses to buccal nifedipine in patients with this disease, a gas chromatographic assay was used to measure blood nifedipine levels, which were then correlated with the hemodynamic effects.

Nifedipine levels plateaued 20 minutes after the administration of either 10 or 20 mg buccally and did not significantly increase even with the administration of a second 10 mg dose. The values were similar, independent of whether 10 or 20 mg was administered as the first dose. As expected, there was a linear relationship between nifedipine concentrations and increases in heart rate and decreases in peripheral vascular resistance. Cardiac output increased only as a result of heart rate changes as stroke volume remained the same initially and then significantly decreased at peak nifedipine levels. Pulmonary wedge pressure was also linearly related to nifedipine levels. When patients were divided on the basis of their control wedge pressure, those with initially normal values had a significant increase in wedge pressure as drug levels rose, with the maximum increase being 51$\pm$26% ($p<0.005$). There was a tendency for patients who started with an elevated wedge pressure to increase their values, but the increase did not achieve statistical significance.

In contrast to our results with verapamil administration, left ventricular outflow tract obstruction was not affected at lower nifedipine levels (<80ng/ml), but actually increased (14$\pm$26%, $p<0.05$) at levels above this concentration. Also different from the verapamil findings was the fact that nifedipine had no effect on radionuclide angiographic determined indicators of systolic and diastolic left ventricular function.

Thus we were not able to demonstrate any beneficial hemodynamic effects of buccally administered nifedipine to patients with hypertrophic cardiomyopathy. Moreover, at higher concentrations of the drug, there was a tendency for detrimental effects to occur, a reduction in stroke volume, an increase in left ventricular outflow tract obstruction, and an elevation of left ventricular filling pressures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04027-03 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Angina caused by reduced vasodilator reserve of the small coronary arteries.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Martin B. Leon, M.D.	Sr. Investigator	CB NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To study the mechanism of chest pain in patients with arteriographically normal coronary arteries, 50 patients underwent great cardiac vein flow, lactate and oxygen content determination at rest and during pacing, and left ventricular end-diastolic pressure measurement immediately after pacing. Those patients experiencing their typical chest pain during pacing had less of a rise in flow and fall in coronary resistance, less lactate consumption, and higher post-pacing left ventricular end-diastolic pressure than those patients without pain. After ergonovine, a larger group demostrated hemodynamic abnormalities similar to patients experiencing chest pain in the control pacing study. No epicardial coronary artery spasm was noted after ergonovine. The data suggest that some patients with chest pain and angiographically normal appearing coronary arteries have dynamic abnormalities of the small coronary arteries or coronary microcirculation, resulting in abnormal vasocilator reserve or vasoconstriction.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

To study the mechanism of chest pain in patients with angiographically normal-appearing coronary arteries, 50 such patients underwent great cardiac vein (GCV) flow, oxygen content, and lactate determination at rest and during pacing, and left ventricular end-diastolic pressure (LVEDP) measurements at rest and immediately after pacing.

Rest

	HR	BP	GCV Flow	CR
No chest pain (n=26)	73±10	97±14	68±16	1.52±.45
Chest pain (n=24)	79±10	99±16	72±18	1.45±.45

Pacing

	HR	BP	GCV Flow	CR
No chest pain (n=26)	150±0	102±14	124±27	.86±.25
Chest pain	148±6	109±17	98±26*	1.20±.37

*=$p<0.001$ vs. no chest pain group. Thus, the 24 patients experiencing their typical chest pain during pacing demonstrated less of a rise in coronary flow and less of a fall in coronary resistance (CR) compared to those patients without chest pain. Lactate consumption at heart rate 150 was significantly less (28.3±21.5 vs 51.3±35.8 mM.ml/min, $p<0.001$, and the increase in LVEDP from rest to immediately after pacing significantly greater (5±2 vs 1±2 mm/tg, $p<0.001$ in the chest pain group.

After administration of ergonovine 0.15, iv to 46 of these patients, 31 developed their typical chest pain during pacing. This group demonstrated significantly less increase in flow (38±20% vs. 107±38%, $p<0.001$), and fall in coronary resistence (-16±12% vs. -45±11%, $p<0.001$) compared to the 15 patients not developing chest pain, despite no significant difference in MVO_2 between the 2 groups. Patients developing pain also exhibited lower lactate consumption at heart rate 150 (39.2±23.6 vs. 65.3±46.3 mm.ml/min, $p<0.01$), greater arterial - GCV O_2 difference (12.5±1.3 vs. 11.6±1.0 ml. O_2/100 ml, $p<0.05$) and a more marked increase in LVEDP from rest to post pacing (11±3 vs. 5±2 mm Hg, $p<0.001$). Quantitative coronary angiography demonstrated no significant luminal narrowing of the epicardial coronary arteries in response to ergonovine.

These data are consistant with the hypothesis that some patients with chest pain and angiographically normal-appearing coronary arteries have dynamic abnormalities of the small coronary arteries or coronary microcirculation that cause abnormal vasodilator reserve or vasoconstriction resulting in myocardial ischemia and angina pectoris.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04045-02 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Left ventricular dysfunction in patients with angina pectoris.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Robert O. Bonow, M.D.	Sr. Investigator	CB	NHLBI
Stephen L. Bacharach, Ph.D.		NM	CC
Michael V. Green, M.D.	Chief, Applied Physics Sec.	NM	CC
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Martin B. Leon, M.D.	Sr. Investigator	CB	NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Much debate has focused on whether or not patients with chest pain despite normal epicardial coronary arteries truly experience myocardial ischemia. Fifty such patients underwent both a pacing coronary flow study and rest and exercise gated blood-pool scintigraphy. During atrial pacing those patients developing their typical chest pain, especially after ergonovine administration, demonstrated apparent abnormal vasodilator reserve of the coronary circulation, associated with mechanical and metabolic evidence of ischemia. The 40 patients with abnormal vasodilator reserve demonstrated reduced rest and exercise left ventricular ejection fraction by gated blood pool scintigraphy, and half the patients in this group demonstrated exercise-induced wall motion abnormalities. These patients also manifested impaired diastolic filling at rest. Thus, patients with chest pain due to abnormal vasodilator reserve demonstrate abnormalities of left ventricular systolic and diastolic function suggestive of myocardial ischemia.

Project Description:

Fifty patients with chest pain despite angiographically normal coronary arteries, underwent both a pacing coronary flow study and rest and exercise gated blood-pool scintigraphy. During atrial pacing to heart rate 150, those patients developing their typical chest pain demonstrated significantly lower great cardiac vein flow, higher coronary resistence, and less lactate consumption than those without pain.

Control Pacing Study:

		GCV Flow	CR	Lactate Consumption
No chest pain	n=24	135±57	.85±.29	42.4±23.6
Developing chest pain	n=26	102±25*	1.11±.33*	24.2±20.7*

*=p<0.005 vs. no chest pain, GCV=great cardiac vein (ml/min), CR=coronary resistence ($\frac{\text{mean blood pressure}}{\text{GCV flow}}$), lactate consumption (mM.ml/min)

Administration of ergonovine 0.15 intravenously prior to repeat atrial pacing resulted in chest pain and limited vasodilitation in a larger number of patients than pacing alone, without significant epicardial coronary artery luminal narrowing.

Ergonovine Pacing Study:

No chest pain (n=11)	135±32	.88±.25	56.0±34.7
Developing chest pain (n=35)	105±38**	1.20±.41**	33.8±22.9**

**=p<0.025 vs. no chest pain.

A total of 40 patients were considered to have abnormal vasodilator reserve on the basis of these studies. The results of gated blood-pool scintigraphy in these patients were compared to the remaining 10 patients, and 52 age and sex-matched controls free of known heart disease.

	Rest EF	Exercise EF	Chest Pain
Controls (n=52)	57±8	66±10	0/52
NVR (n=10)	51±5*	63±3	5/10
AVR (n=40)	51±10**	55±13**	22/40

*=p<0.01, **=p<0.005 vs. controls, EF=ejection fraction, NVR=normal vasodilator reserve, AVR=abnormal vasodilator reserve.

Parameters of left ventricular systolic function

	Rest EF	Exercise EF		WMA		Total
	<45%	EF<0%	EF<5%	Rest	Exercise	Abnormalities
Control (n=52)	3	4	9	1	1	12/52(23%)
NVR (n=10)	1	0	2	0	0	3/10(30%)
AVR (n=40)	8	9	23	6	19	27/40(68%)
P vs. controls	.04	.04	<.001	.05	<.001	<.001
P vs NVR	NS	NS	.03	NS	.02	.07

WMA=wall motion abnormalities; Total abnormalities=number of patients in each group with 1 or more abnormality in systolic left ventricular function.

Patients with abnormal vasodilator reserve also manifested impaired left ventricular diastolic filling at rest compared to controls (peak filling rate 2.3±0.8 vs. 3.2±0.7 end diastolic volume/sec, $p<0.005$).

Thus, patients with chest pain due to abnormal vasodilator reserve demonstrate abnormalities of left ventricular systolic and diastolic function suggestive of myocardial ischemia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04067-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detrimental effect of ergonovine in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2	.4	1.6

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with hypertrophic cardiomyopathy(HCM) frequently experience chest pain that occurs with variable threshold of onset and is often prolonged in duration. This study was designed to evaluate the effect of a pharmacologic vasoconstrictor agent, ergonovine, on the coronary vasculature of patients with HCM. Fifteen patients with HCM and chest pain despite angiographically normal appearing coronary arteries underwent a pacing study with measurement of coronary flow, and myocardial mechanics and metabolism. Afterward, a repeat pacing study was performed after administration of ergonovine. HCM patients were found to have high resting coronary flow as expected due to the increased muscle mass associated with this disease. Eight HCM patients demonstrated a fall in coronary flow at high paced heart rates after an initial rise in flow at lower paced heart rates. This fall in flow was associated with a striking elevation in left ventricular filling pressure and metabolic evidence of ischemia. These same patients demonstrated vasoconstriction of the small coronary arteries or arteriole during pacing after ergonovine. This sensitivity to a vasoconstrictor stimulus (possibly because coronary arterioles are maximally dilated) may explain the variation in anginal threshold many HCM patients experience.

Project Description:

Many patients with hypertrophic cardiomyopathy (HCM) have high resting coronary flow, but during pacing coronary flow often falls at high paced heart rates (HR 150), compared to intermediate paced heart rates (HR 120-130). To study whether vasoconstrictor stimuli effect flow dynamics, great cardiac vein flow (ml/min) was measured at rest and during pacing, before and after ergonovine .15 mg iv. Group A=8 patients whose flow fell at high paced heart rates, and Group B=7 patients without a fall in flow. Coronary blood flow was estimated by measurement of great cardiac vein (GCV) flow, reflecting flow in the anterior coronary circulation. Data=mean ±SD

	Rest		Mid-Pacing		High-Pacing	
	BP	GCV Flow	BP	GCV Flow	BP	GCV Flow
A	91±15	102±28	95±16	143±21	98±12	115±29
B	104±21	75±15*	113±23	111±49	116±27	124±47

(*=p<0.025 vs A). Post pacing LVEDP was higher in Group A (36±7mm Hg vs.24±8, p<0.05).
After ergonovine:

	Rest		Mid-Pacing		High-Pacing	
	BP	GCV Flow	BP	GCV Flow	BP	GCV Flow
A	102±16	108±36	104±16	119±29**	108±18	113±31
B	113±22	88±33	121±23	116±35	126±24	135±45

(**=p<0.025 vs control Group A mid P F).

All blood pressures (BP) were significantly higher after ergonovine compared to control pacing. The post-pacing left ventricular end-diastolic pressure (LVEDP) was higher in Group A (35±7 mm Hg vs 27±7, p<0.025). Coronary arteriography after ergonovine showed no significant coronary artery narrowing.

Thus, in many HCM patients with high rest coronary flow, flow increases with moderate increase in HR and then decreases at higher HR, possibly due to ischemia-induced elevation in LVEDP. Peak coronary flow in these patients decreases with ergonovine. This sensitivity to a vasoconstrictor stimulus (possibly because coronary arterioles are maximally dilated) may explain variation in anginal threshold many HCM patients experience.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-HL-04068-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study examining myocardial ischemia in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Douglas R. Rosing,M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Barry J. Maron, M.D.	Head, Echo Lab	CB	NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB	NHLBI
Robert O. Bonow, M.D.	Head, Radionuclide Lab	CB	NHLBI
Rita M.Watson, M.D.	Sr. Medical Staff Fellow	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

x (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

_ (a1) Minors

_ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chest pain is a frequent symptom of patients with hypertrophic cardiomyopathy (HCM), commonly occurring in the setting of angiographically normal epicardial coronary arteries. In contrast to the generally reproducible effort angina of patients with coronary artery disease, HCM patients often report pain with variable threshold of onset, often prolonged in duration even after cessation of activity. Twenty patients with HCM and chest pain despite angiographically normal coronary arteries underwent a pacing study with measurement of great cardiac vein flow, lactate and oxygen content in addition to left ventricular filling pressure. A cohort of 28 patients without HCM who underwent the same pacing study without experiencing chest pain served as controls.

During pacing coronary flow rose in both groups although coronary and myocardial hemodynamics differed greatly. In contrast to the linear increase in flow in control up to a heart rate of 150, HCM patients demonstrated an initial rise in flow at an intermediate heart rate, at which point 12 of 20 HCM patients described their typical chest pain. Continued pacing to a heart rate of 150 resulted in a fall in coronary flow in 14 of 20 HCM patients, associated with a substantial rise in left ventricular filling pressures and metabolic evidence of ischemia. A paradoxical narrowing of the arterial-venous oxygen difference was also noted, which may contribute to ischemia. Thus, most HCM patients achieve maximum coronary vasodilatation at modest increases in heart rate. Myocardial ischemia occurs at this point, resulting in elevation in left ventricular filling pressures, probably because of ischemia-related changes in ventricular compliance, with a subsequent adverse effect on coronary blood flow.

Project Description:

To study the mechanism and hemodynamic significance of myocardial ischemia in hypertrophic cardiomypathy, 20 patients (9 with resting left ventricular outflow obstruction ≥30 mmHg) with a history of angina pectoris and angiographically normal coronary arteries underwent a pacing study with measurement of great cardiac vein flow, lactate and oxygen content in addition to left ventricular filling pressure. Twenty-eight patients without hypertrophic cardiomyopathy underwent an identical pacing study without experiencing chest pain and serve as controls. Data expressed as mean ±SD.

	Rest			Mid-Pacing			Peak Pacing		
	HR	BP	GCV-F	HR	BP	GCV-F	HR	BP	GCV-F
Controls (n=28)	72±10	97±13	66±17	130±0	101±13	107±34	150±0	103±15	128±28
HCM (n=20)	82±12	94±13	91±27	127±13	102±19	133±31*	147±7	104±21	114±29**

* = p>0.001 vs. control, ** = p<0.001 vs. HCM mid-pacing.
GCV-F = great cardiac vein flow (ml/min), BP = mean arterial blood pressure (mmHg).

	Rest			Peak Pacing		
	AVO_2	MVO_2	Lactate	AVO_2	MVO_2	Lactate
Controls	12.3±1.7	8.3±2.7	26.5±19.1	11.5±1.5	13.9±4.0	54.5±34.9
HCM	11.7±1.8	10.5±3.8***	27.2±26.3	10.9±2.1	12.4±3.5	6.9±32.6*

*** = p<0.025, * = p<0.001 vs. controls

AVO_2 = arterial-GCV O_2 difference ($ml O_2$/100 ml), MVO_2 = myocardial oxygen consumption (ml/min), lactate = lactate consumption (mM.ml/min).

	Rest LVEDP	Post-pacing LVEDP
Controls	11±3	12±4
HCM	16±6*	30±9*

* = p<0.001 vs. controls. LVEDP = left ventricular end-diastolic pressure.

In contrast to the linear increase in coronary flow in control up to a heart rate of 150, HCM patients demonstrated an initial rise in flow at an intermediate heart rate, associated with 12 of 20 patients experiencing their typical chest pain. With continued pacing to heart rate 150, coronary flow actually fell in 14 HCM patients, with 18 of 20 patients experiencing chest pain and metabolic evidence of myocrdial ischemia. This fall in coronary flow was associated with a substantial rise in LVEDP. Despite metabolic and hemodynamic evidence of myocardial ischemia, the AVO_2 difference actually narrowed at peak pacing.

Thus, patients with HCM have a high resting flow, in part related to increased muscle mass (estimated by 2-D echocardiography, and left ventricular outflow obstruction. Most patients achieve maximum vasodilation and flow at modest increases in heart rate, associated with

the onset of angina at higher paced heart rates. Elevation in left ventricular filling pressure, probably related to ischemia-induced changes in ventricular compliance, is associated with a decline in coronary flow. A paradoxical narrowing of the AVO_2 difference, despite apparent coronary flow limitation may also be of pathogenetic importance to myocardial ischemia in HCM.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04069-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intraoperative 2-D echocardiography in HCM during septal myotomy-myectomy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echo Lab	CB	NHLBI
Charles McIntosh, M.D.	Senior Surgeon	SU	NHLBI
Javier Arce, M.D.	Medical Technician	CB	NHLBI
Yvonne Wesley	Medical Technician	CB	NHLBI

COOPERATING UNITS *(if any)*

Heart Surgery Branch, NHLBI

LAB/BRANCH

Cardiology Branch

SECTION

Echocardiographic Section

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ventricular septal myotomy-myectomy operation for obstructive hypertrophic cardiomyopathy requires intimate knowlege of ventricualr septal anatomy for successful outcome. Often routine echocardiography can not provide reliable information regarding septal morphology because of technical limitations. Therefore, we utilized a sterilizable 5MHz two-dimensional echo transducer for intraoperative studies in 9 patients with hypertrophic cardiomyopathy; echos were performed by placing the transducer directly on the anterior surface of right ventricle. In 6 of 9 patients, preoperative transthoracic M-mode and 2-dimensional echos did not provide definitive assessment of ventricular septal thickness. In each of these patients, measurements of ventricular septal thickness varied by 5-11 mm (average 7) due to suboptimal visualization of ventricular septal endocardial surfaces. However, in each patient intraoperative echocardiography provided a clear definition of endocardial borders and more accurate definition of ventricular septal thickness. In one patient transthoracic echo underestimated ventricular septal thickness by 5 mm; in the remaining 2 patients ventricular septal thickness by transthoracic and intraoperative echo were similar. Intraoperative echocardiography performed after resection of ventricular septal muscle also provided direct morphologic assessment of the depth, length and width of the ventricular septal myotomy-myectomy resection.

Therefore, our initial experience indicates that intraoperative two-dimensional echocardiography is a valuable aid to the surgeon performing the ventricualr septal myotomy-myectomy operation.

144

Project Description:

Ventricular septal myotomy-myectomy is recommended to patients with obstructive hypertrophic cardiomyopathy who do not benefit from medical therapy for the purpose of relieving subaortic obstruction . Ventricular septal myotomy-myectomy is performed through an aortotomy which prohibits the surgeon from directly visualizing the entire operative site. Thus, precise knowledge of ventricular septal anatomy is crucial to successful operation. In order to better define septal morphology, we utilized a sterilizable Diasonics 5MHz two-dimensional echo transducer for intraoperative studies in 9 patients with hypertrophic cardiomyopathy; echos were performed by placing the transducer directly on the anterior surface of right ventricle. In 6 of 9 patients, preoperative transthoracic M-mode and 2-dimensional echos did not provide definitive assessment of ventricular septal thickness. In each of these patients, measurements of ventricular septal thickness varied by 5-11 mm (average 7) due to suboptimal visualization of ventricular septal endocardial surfaces. However, in each patient intraoperative echocardiography provided a clear definition of endocardial borders and more accurate definition of ventricular septal thickness. In one patient transthoracic echo underestimated ventricular septal thickness by 5 mm; in the remaining 2 patients ventricular septal thickness by transthoracic and intraoperative echo were similar. Intraoperative echo performed after resection of ventricular septal muscle also provided direct morphologic assessment of the depth, length and width of the ventricular septal myotomy-myectomy resection. In 8 patients intraoperative echocardiography showed ventricular septal myotomy-myectomy to be adequate and mitral systolic anterior motion was abolished or greatly reduced; in the other patient ventricular septal myotomy-myectomy was narrow and shallow and systolic anterior motion persisted. Hence, intra-operative 2-dimensional echocardiography is a rapid and easily performed procedure which constitutes a valuable aid and guide to the surgeon performing ventricular septal myotomy-myectomy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04070-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Left ventricular filling by pulsed Doppler echocardiography in HCM

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Barry J. Maron, M.D.	Head, Echo Lab	CB NHLBI
Javier Arce, M.D.	Medical Technician	CB NHLBI
Robert O. Bonow, M.D.	Sr. Investigator	CB NHLBI
Yvonne Wesley	Medical Technician	CB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Echocardiographic Section

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.4	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There are few truly non-invasive tests available to measure left ventricular diastolic function in patients with cardiac disease. In this study we utilized pulsed Doppler to assess transmitral flow-velocity patterns and characterize left ventricular filling and relaxation in 67 patients with hypertrophic cardiomyopathy and 35 normal controls. Patients with hypertrophic cardiomyopathy differed distinctly from normals with prolonged rapid diastolic filling, shortened diastasis, enhanced filling during atrial systole, (reduced ratio of peak flow-velocity in early diastole to that in atrial systole) and prolonged isovolumic relaxation. Abnormal left ventricular filling was present in 48 (72%) of 67 patients with hypertrophic cardiomyopathy, but was more common in symptomatic (25/27, 93%) than asymptomatic patients (23/40; 57%; $p<0.01$) and more frequent in obstructive (17/19, 90%) than in nonobstructive hypertrophic cardiomypathy (31/48, 65%; $p<0.05$).

These findings demonstrate that pulsed Doppler echocardiography may be used to quantitatively assess left ventricular function and that impairment in left ventricular filling and relaxation are common and clinically important abnormalities in a population of patients with hypertrophic cardiomyopathy.

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Project Description:

Analysis of trans-mitral valve flow-velocity may provide useful data on left ventricular diastolic function. We utilized pulsed Doppler echocardiography to record trans-mitral valve flow-velocity in 67 untreated patients with hypertrophic cardiomyopathy and 35 normal controls of similar age and heart rate. Patients with hypertrophic cardiomyopathy differed distinctly from normals with prolonged rapid diastolic filling, shortened diastasis, enhanced filling during atrial systole (reduced ratio of peak flow-velocity in early diastole to that in atrial systole) and prolonged isovolumic relaxation.

Abnormal left ventricular filling was present in 48 (72%) of 67 patients with hypertrophic cardiomyopathy, but was more common in symptomatic (25/27, 93%) than asymptomatic patients (23/40; 57%; $p<0.01$) and more frequent in obstructive (17/19, 90%) than in nonobstructive hypertrophic cardiomyopathy (31/48, 65%; $p<0.05$). Hence, 1) pulsed Doppler echocardiography is a new and valuable noninvasive method for quantitatively assessing left ventricular diastolic function, 2) left ventricular diastolic abnormalities are common (72%) in hypertrophic cardiomyopathy, but most frequent in patients with symptoms or subaortic obstruction. These findings underline the frequency and importance of impaired left ventricular filling in a population of patients with hypertrophic cardiomyopathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04071-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Spontaneous progression of left ventricular hypertrophy during adolescence in HCM

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echo Lab	CB NHLBI
Javier Arce, M.D.	Medical Technician	CB NHLBI
Yvonne Wesley	Medical Technician	CB NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Echocardiographic Section

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Twenty-one relatives of patients with documented hypertrophic cardiomyopathy were studied prospectively over a period of 5 years with M-mode and wide-angle two-dimensional echocardiography to evaluate the potential for progression of left ventricular hypertrophy. Although each of these subjects had no or minimal hypertrophy prior to adolescence, 11 showed marked increase in the magnitude and distribution of hypertrophy including 3 who progressed from a normal heart. These findings also emphasize that echocardiographic screening of pre-adolescent family members cannot definitively exclude hypertrophic cardiomyopathy.

145

Project Description:

Hypertrophic cardiomyopathy has been considered to be a congenital cardiac malformation with left ventricular hypertrophy often evident shortly after birth. To determine whether left ventricular mass is altered early in life, 21 asymptomatic and untreated relatives of patients with hypertrophic cardiomyopathy were studied serially with 1 and 2-dimensional echo; initially at age 8-15 years (mean 11) and most recently at 13-19 years (mean 15). At initial examination, 13 patients had structurally normal hearts and 8 had mild left ventricular wall thickening. Over a 3-5 year follow-up, 11 patients showed marked increase in the magnitude and in the distribution of left ventricular hypertrophy, including 3 patients with progression from a normal left ventricle (at ages 9-12) to wall thickness of 14-21 mm (ages 13-17). Left ventricular wall thickness increased as much as 150% (average 75%), from 13.8±4 mm to 24.4±1 mm ($p<0.001$). This increase was in the basal anterior septum (5 patients) or areas of left ventricle not letectable by M-mode echo - i.e., anterior free wall (3 patients), posterior or distal ventricular septum (3 patients). In each patient progression of left ventricular hypertrophy occurred during a period of considerable body growth, at 12-19 years of age, but was not due to left ventricular pressure overload (mitral systolic anterior motion was absent). In conclusion, we have shown for the first time, that the morphologic expression of nonobstructive hypertrophic cardiomyopathy can spontaneously appear or progress substantially in genetically predisposed relatives during adolescence when body growth is accelerated. These findings also emphasize that echocardiographic screening of pre-adolescent family members cannot definitively exclude hypertrophic cardiomypathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04072-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Obstruction in hypertrophic cardiomyopathy: analysis by Doppler echocardiography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echo Lab	CB	NHLBI
John S. Gottdiener, M.D.	Guest Investigator	CB	NHLBI
Javier Arce, M.D.	Medical Technician	CB	NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Yvonne Wesley	Medical Technician	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Echocardiographic Lab

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A major controversy persists in cardiology as to whether true outflow obstruction may exist in patients with hypertrophic cardiomyopathy and subaortic gradients. In this study range-gated Doppler echocardiography was utilized to investigate this problem in 50 patients with hypertrophic cardiomyopathy and 20 normals.

In obstructive hypertrophic cardiomyopathy, left ventricular ejection was characterized by early and rapid emptying (76±14% of aortic flow-velocity in the initial one-third of systole). The proportion of forward flow occurring after mitral-septal contact (and therefore concommitant with the gradient and elevated intraventricular pressure) was considerable, averaging over 40%.

Mid-systolic impedance to left ventricular outflow was suggested by the rapid deceleration in aortic flow-velocity concommitant with mitral-septal contact and premature partial aortic valve closure. Furthermore, left ventricular ejection was prolonged (384±40 msec) and the ventricle continued to empty and shorten during the period when both the pressure gradient and markedly elevated intraventricular pressures were present.

In contrast, patients with nonobstructive hypertrophic cardiomyopathy showed no evidence of impedance to left ventricular ejection. Aortic flow-velocity waveforms were similar to normals, with flow persisting to aortic valve closure; significant systolic anterior motion and partial mid-systolic aortic valve closure were absent, and the systolic ejection period was normal (303 ± 27msec).

Hence, in patients with hypertrophic cardiomyopathy mitral valve systolic anterior motion constitutes the mechanical obstruction to left ventricular emptying. Gradients produced by this mitral valve motion appear to be of pathophysiologic importance since the left ventricle continues to contract in the presence of markedly elevated intraventricular pressures.

/ 50

Project Description:

To determine whether true obstruction to left ventricular ejection exists in patients with hypertrophic cardiomyopathy and subaortic gradients, we analyzed with pulsed Doppler echocardiography the patterns of left ventricular emptying in 50 patients with hypertrophic cardiomyopathy (20 with and 30 without evidence of obstruction) and in 20 normals. In obstructive hypertrophic cardiomyopathy, left ventricular ejection was characterized by early and rapid emptying (76±14% of aortic flow-velocity in the initial one-third of systole). The proportion of forward flow-velocity occurring before initial mitral-septal contact (and, hence, before onset of the subaortic gradient) was variable, but averaged 58%. Conversely, the proportion of forward flow occurring after mitral-septal contact (and therefore concommitant with the gradient and elevated intraventricular pressure) was considerable, averaging over 40%.

Mid-systolic impedance to left ventricular outflow was suggested by the rapid deceleration in aortic flow-velocity concommitant with mitral-septal contact and premature partial aortic valve closure. Furthermore, left ventricular ejection was prolonged (384±40 msec) and the ventricle continued to empty and shorten during the period when both the pressure gradient and markedly elevated intraventricular pressures were present. In 16 of 20 patients a relatively small second peak in flow-velocity appeared in late systole. Since marked systolic anterior motion of the mitral valve was still present, the late systolic portion of forward flow-velocity also appeared to be largely ejected during imposition of a mechanical impediment to outflow.

In contrast, patients with nonobstructive hypertrophic cardiomyopathy showed no evidence of impedance to left ventricular ejection. Aortic flow-velocity waveforms were similar to normals, with flow persisting to aortic valve closure; significant systolic anterior motion and partial mid-systolic aortic valve closure were absent, and the systolic ejection period was normal (303 ± 27 msec).

We conclude that in hypertrophic cardiomyopathy: 1) mitral valve systolic anterior motion produces a mechanical obstruction to left ventricular emptying, and a considerable portion of the stroke volume is impeded in its egress from the left ventricle; 2) gradients appear to be of pathophysiologic importance since the left ventricle continues to contract in the presence of markedly elevated intraventricular pressures; and 3) left ventricular ejection characteristics differ markedly between patients with and those without subaortic gradients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04073-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ancrod in patients with refractory angina: angiographic findings

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
Richard O. Cannon, III, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To test the hypothesis that thrombus may play a role in some coronary artery disease patients without acute myocardial infarctions, Ancrod, a snake venom derivative with defibrinogenating and indirect tissue plasminogen activator properties, was given daily for 2 weeks to 10 patients with refractory angina. Coronary angiography and great cardiac vein flow studies at rest and during pacing were performed before and after Ancrod therapy to assess anatomic changes, coronary vasodilator reserve, and metabolic indices of ischemia. During coronary cineangiography, before Ancrod therapy, visible thrombi were absent in all patients. After Ancrod therapy, no instances of recannalization were seen in totally occluded bypass grafts (n=17) or native vessels (n=16). Lumen caliber changes in subtotal stenoses were also not affected by Ancrod therapy, employing quantitative coronary cineangiographic techniques. Moreover, Ancrod therapy did not improve great cardiac vein flow with pacing or metabolic indices of ischemia, including changes in arterial venous oxygen consumption and transmyocardial lactate metabolism. However, all patients increased exercise capacity greater than 50% and 3 patients had marked reduction in angina. Thus, it appears that Ancrod therapy may improve exercise capacity and reduce angina in some patients without changing objective parameters such as coronary anatomy, great cardiac vein blood flow, or metabolic indices of ischemia. This suggests that coronary thrombi were not importantly contributing to symptoms in these patients. Hence, the beneficial clinical responses to Ancrod may be related to altered blood viscosity or as yet undetermined mechanisms.

Project Description

To test the hypothesis that thrombus may play a role in some coronary artery disease patients without acute myocardial infarction, Ancrod, a snake venom derivative with defibrogenating and indirect tissue plasminogen activator properties, was given daily for 2 weeks to 10 patients with refractory angina. Coronary angiography and great cardiac vein flow studies at rest and during pacing were performed before and after Ancrod to assess anatomy, vasodilator reserve, and metabolic indices of ischemia. During coronary angiography, before Ancrod, visible thrombus was absent in all patients; after Ancrod, no instances of recanalization were seen in totally occluded bypass grafts (n=17) or native vessels (n=16). Lumen caliber changes in subtotal stenoses were also not affected by Ancrod. Data = mean ± 1 standard deviation.

	Percent Change Great Cardiac Vein Flow	Peak Pressure Rate Product With Pacing	Arteriovenous Oxygen Content Difference (ml O_2/100ml)	Arteriovenous Lactate Consumption Difference (mM.ml/min)
Before Ancrod	37±29	16.0±2.0	-.014±2.4	-21.4±40.6
After Ancrod	36±22	17.2±2.8	.37±1.2	-12.5±3.5

Although Ancrod did not improve coronary angiography, great cardiac vein flow with pacing, or metabolic indices, all patients increased exercise capacity >50% and 3 patients had marked reduction in angina. Thus, Ancrod therapy improved exercise and reduced angina in some patients without changing coronary angiography, great cardiac vein flow, or metabolic indices, suggesting that coronary thrombus was not importantly contributing to symptoms. Hence, the beneficial clinical responses to Ancrod may be related to altered blood viscosity or as yet undetermined mechanisms.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04074-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Amiodarone in patients with hypertrophic cardiomyopathy and refractory symptoms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Barry J. Maron, M.D.	Head, Echo Lab	CB NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB NHLBI
Larry J. Lesko, Ph.D.	Pharmacokinetics Laboratory	Univ of Md
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS *(if any)*

Pharmacokinetics Laboratory, University of Maryland
Nuclear Medicine Department, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.20	0.20	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
  - [ ] (a1) Minors
  - [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An increasing subgroup of patients with hypertrophic cardiomyopathy and refractory cardiac symptoms, despite treatment with standard medical therapies and surgery (whenever possible) has provided the impetus for investigating new forms of drug therapies. Amiodarone, a benzofurane compound, with potent antiarrhythmic properties, would appear to have a hemodynamic profile suitable in treating the physiologic abnormalities associated with hypertrophic cardiomyopathy. As such, 10 patients with hypertrophic cardiomyopathy and refractory cardiac symptoms were treated with high dose oral amiodarone (800 to 1600 mg per day for 10 days) and was compared with no medications and previous best medical therapy (including calcium channel blockers and beta blockers). Therapeutic efficacy was assessed by exercise testing, ambulatory ECG monitoring, and semiquantitative symptom scores. Exercise duration increased greater than 100% from control values in 7 of 10 patients. The improvement correlated poorly with changes in heart rate, other resting or exercise hemodynamic parameters, QT interval and amiodarone blood levels. Ventricular tachycardia during ambulatory monitoring was present in 6 patients on no medication, or best medical therapy, but was absent with amiodarone; nevertheless, one patient died suddenly 5 weeks after beginning amiodarone therapy. We conclude that high dose oral amiodarone, unrelated to its antiarrhythmic effects, improves exercise capacity and cardiac symptoms in many patients with hypertrophic cardiomyopathy and may be used as an alternative to current medical therapy in patients with refractory symptoms. However, the long term efficacy of amiodarone remains unknown.

Project Description:

To determine the usefulness of amiodarone in patients with hypertrophic cardiomypathy and refractory cardiac symptoms, high dose oral amiodarone (300-1600 mg/day for 10 days) was compared with no medications and "best" medical therapy (best medical therapy = verapamil, nifedipine and/or beta blockers) in 10 patients, who were functional class 3 or 4. Efficacy was assessed by exerise testing, ambulatory ECG-monitoring, and semiquantitative cardiac symptom scores. Data = mean±1 standard deviation.

	Heart Rate	Blood Pressure	Exercise Duration (secs)	Symptom Score	Premature Ventricular Complex
No medications	78±18	111±14/69±10	209±174	-	263±585
Best Medical Therapy	75±13	109±12/68±9	232±162	36±11	53±79
High dose oral Amiodarone	69±10*	113±13/70±10	442±207**+	22±12++	6±14*

*p<.05, **p<.005 vs no medications; +p<.05, ++p<.005 vs verapamil, nifedipine and/or beta blockers.

Exercise duration increased >100% from no medication values in 7/10 patients. The improvement correlated poorly with change in heart rate, other resting or exercise hemodynamic parameters, QT interval and A blood levels. Ventricular tachycardia during ambulatory monitoring was present in six patients on no medications or during best medical therapy, but was absent with amiodarone; nevertheless, 1 patient died suddenly 5 weeks after beginning amiodarone therapy. We conclude that high dose amiodarone, unrelated to its antiarrhythmic effects, improves exercise capacity and cardiac symptoms in some patients with hypertrophic cardiomypathy and may be used as an alternative to current medical therapy in patients with refractory symptoms. However, the long-term efficacy of amiodarone is unknown.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04075-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ancrod in chronic angina: effects on exercise and ventricular function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB NHLBI
Richard O. Cannon, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB NHLBI

COOPERATING UNITS *(if any)*

Nuclear Medicine Department, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To determine the potential contribution of coronary thrombus to severe chronic stable angina, thrombolytic therapy, using Ancrod, a snake venom derivative with defibrinogenating and indirect tissue plasminogen activator properties was given to 10 patients. After stopping medications, patients were randomized and treated blindly with either daily infusions of placebo or Ancrod for 2 weeks. Patients given placebo first were then given open-label Ancrod for 2 more weeks. Efficacy was assessed using exercise tests and radionuclide cineangiograms at rest and during matched levels of exercise. During Ancrod therapy, all patients increased exercise duration greater than 50% compared with pre-study control values and 3 patients with severe rest angina became symptom free. However, exercise duration was not associated with improved ST-segment changes, peak pressure rate product during exercise, or reduced radionuclide cineangiographic indices of ischemia. These data suggest that thrombolytic therapy using Ancrod in refractory angina improves exercise capacity in most patients, but the mechanism underlying beneficial responses and long-term efficacy remain undefined. This study is the first attempt to examine the possible role of thrombus formation in coronary syndromes other than acute myocardial infarction and the results should will stimulate much needed additional work.

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Project Description:

To determine the contribution of coronary thrombus to severe chronic stable angina, thrombolytic therapy using Ancrod, a snake venom derivative with defibrinogenating and indirect tissue plasminogen activator properties was given to 10 patients. After stopping medications, patients were randomized and treated blindly with daily infusions of either placebo or Ancrod for 2 weeks; patients given placebo were then given open-label Ancrod for 2 more weeks. Efficacy was assessed using exercise tests and radionuclide angiograms at rest and during matched exercise.

	(n)	Exercise Duration	Peak Exercise Pressure Rate	Rest Ejection Fraction	Change in Ejection Fraction	Rest Peak Filling Rate
Before Ancrod	(10)	113±59	17.8±3.3	45±12	-6±6	1.7±.6
Placebo	(5)	170±136	15.0±4.3	45±13	-8±13	1.9±.7
Blinded Ancrod	(5)	183±71*	17.5±3.5	42±10	-8±10	2.2±1.0
All Ancrod	(10)	302±207**	18.3±5.6	40±9	-4±8	1.9±.8

Data=mean± 1 standard deviation. *p<.005 vs. matched control, **=p<.005 vs. before Ancrod.

During Ancrod therapy, all patients increased exercise duration >50% compared with before Ancrod and 3 patients with severe rest angina became symptom-free. However, exercise duration was not associated with improved ST segment changes, peak exercise pressure rate during exercise, or reduced radionuclide cineangiographic indices of ischemia. These data suggest that thrombolytic therapy using Ancrod in refractory angina improves exercise capacity in most patients but the mechanisms underlying beneficial responses and long-term efficacy remain undefined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04076-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assessment of laser-tissue interactions in human cadaver coronary arteries

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
David Underhill, M.D.	Clinical Associate	SU NHLBI
Robert Bonner, Ph.D.	Engineer	DRS BEI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB NHLBI
William Roberts, M.D.	Chief, Pathology Br	PA NHLBI

COOPERATING UNITS *(if any)*

Cardiac Surgery Branch, NHLBI; Cardiac Pathology Branch, NHLBI; Biomedical Engineering and Instrumentation Br, DRS; National Aeronautics and Space Admin.; Naval Research Laboratories.

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The potential use of lasers interfaced with optical wave guides and incorporated into catheter systems is a provocative potentially important area of future clinical investigation. However, before such bold instrumentation can be employed in humans, a variety of basic studies helping to clarify correct laser sources and to predict biological effects of laser-tissue interactions are necessary. The present investigation includes an evaluation of four different lasers (Argon, Neodymium-YAG, Carbon Dioxide, and Ultraviolet Excimer) with widely varying physical properties studied during conditions of altered power and pulse duration. Laser effects were studied in dry medium (air) and in solution (saline), with and without appropriate optical wave guides, and tissue specimens employed were fresh human cadaver coronary arteries in both normal patients and patients with known underlying atherosclerosis. Specific laser effects were assessed using thermocouples and fast infrared photography to define thermal diffusion properties and gross pathologic and light microscopic changes. These experiments have helped to differentiate specific effects of given laser sources and will provide important information to be used in subsequent in vivo animal studies.

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Project Description:

There is growing excitement surrounding the area of potential clinical usages of laser-tissue interactions in cardiovascular diseases. Early investigation has suggested the possibility of coupling appropriate laser systems with optical wave guides into catheters for the purpose of thermovaporization of atherosclerotic lesions. However, a significant body of preliminary investigation is required to define appropriate laser sources and to predict the specific laser-biological tissue interactions which might be observed. Therefore, we are attempting to examine and differentiate four laser sources - Argon, Neodymium-YAG, Carbon Dioxide, and Ultraviolet Ecimer - studied during varying power and pulse duration settings. Lasers were examined both with and without appropriate optical wave guides in dry medium (air) and in solution (saline). Fresh specimens of human cadaver coronary arteries in both normal patients and patients with underlying atherosclerosis were examined. To assess laser-tissue effects, thermaldiffusion properties were defined employing thermocouples placed behind the tissues and fast infrared photography. Gross pathologic changes and light microscopic changes were also examined with varying laser sources and operator settings. These investigations have helped to differentiate the major lasers being considered for subsequent investigation and have provided important information which will be used in all future in vivo animal studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04077-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Left ventricular functional changes during amiodarone in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB NHLBI
Cynthia Crawford-Green, M.D.	Clinical Associate	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Br	CB NHLBI

COOPERATING UNITS *(if any)*

Nuclear Medicine Department, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology Section

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To help determine the mechanisms whereby amiodarone (a benzofurane compound) improves cardiac symptoms in patients with hypertrophic cardiomyopathy refractory to standard medical therapy, radionuclide cineangiography at rest and during matched levels of exercise was used to assess left ventricular functional changes. Ten patients were treated with high dose oral amiodarone (800-1600 mg per day for 10 days) and compared to no medications and calcium channel blocker therapy (either verapamil or nifedipine). All patients had refractory symptoms with calcium channel blockers but 8 of 10 were clinical responders to amiodarone (clinical response defined as 100% increased exercise capacity and/or greater than 50% decreased semi-quantitative symptom score). These 8 clinical responders were also compared to another group of patients with hypertrophic cardiomyopathy and similar clinical improvement during treatment with the calcium channel blocker, verapamil. No patient manifested significant changes in left ventricular systolic function (rest ejection fraction, exercise ejection fraction, or ejection rate indices); however, both an increased peak filling rate and a decreased time to peak filling rate (suggesting improved diastolic filling), were seen in 66% of clinical responders during verapamil therapy, but in only 1 of 8 clinical responders with amiodarone therapy. Thus, in patients with hypertrophic cardiomyopathy, diastolic filling changes contribute to clinical improvement during verapamil treatment, but cannot explain the mechanism underlying symptom benefit during amiodarone therapy.

Project Description:

To help determine the mechanisms whereby amiodarone improves cardiac symptoms in patients with hypertrophic cardiomyopathy, radionuclide angiography at rest and during matched exercise was used to assess left ventricular functional changes in 10 patients treated with high dose oral amiodarone (800-1600 mg/day for 10 days) and compared to no medications and calcium channel blocker, (verapamil or nifedipine). All patients had refractory symptoms with calcium channel blockers but 8/10 were clinical responders with amiodarone. (Clinical responders = >100% increased exercise capacity and/or >50% decreased semi-quantitative symptom score); these 8 patients were also compared to another group of hypertrophic cardiomyopathy clinical responders (n=35) treated with verapamil. Data = mean ±1 standard deviation.

	(n)	Rest Ejection Fraction	Exercise Ejection Fraction	Rest Peak Filling Rate	Rest Time to Peak Filling Rate (msec)
Control	(10)	70±14	69±14	2.8±1.0	204±49
Calcium Channel Blockers	(9)	71±12	69±13	3.3±0.7	184±41**
Amiodarone (Clinical Responders)	(8)	74±11	72±11	3.1±1.1	209±52
Verapamil (Clinical Responders)	(35)	71±14	69±10	3.6±1.2*	172±41*

*p<.05 vs C, ** p<.05 vs Amiodarone. Clinical responders (amiodarone and verapamil) manifested no changes in left ventricular systolic function; however, both increased peak filling rate and decreased time to peak filling rate (suggesting improved diastolic filling) were seen in 23/35 (66%) patients with verapamil but only 1/8 patients with amiodarone. Calcium channel blockers caused lesser changes in diastolic function in patients without improved cardiac symptoms. Thus, in patients with hypertrophic cardiomyopathy, diastolic filling changes contribute to clinical improvement during verapamil therapy but cannot explain the mechanism underlying symptom benefit during amiodarone therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04078-01 CB

PERIOD

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Restenosis following percutaneous transluminal coronary angioplasty

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.)

Stewart Levine, M.D.	Fellow	Georgetown U. Med Ctr
Carolyn J. Ewels, B.S.	Research Associate	Georgetown U. Med Ctr
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Kenneth M. Kent, M.D.	Director, Cardiac Cath Labs	Georgetown U. Med Ctr

COOPERATING UNITS (if any)

Georgetown University Medical Center

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

X (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to evaluate the incidence and causes related to the occurrence of coronary artery restenosis after successful percutaneous transluminal coronary angioplasty (PTCA), 251 consecutive patients, who underwent a successful procedure at either the National Institutes of Health or Georgetown University Medical Center, were evaluated. Follow-up coronary angiography took place in 83 of the initial group of 89 successful patients one to eleven months after successful PTCA. Restenosis occurred in 32 patients (36%), with all but two patients having either recurring angina and/or a positive exercise test. The other two patients had significant collateral flow to the vessel with restenosis. Conversely, 39 of 41 asymptomatic patients did not have restenosis. For the entire group of 251 successes with follow-up of at least 6 months (average 21 months), 54% was asymptomatic. In the symptomatic group restenosis was found in 63% of patients. Progression of disease in other vessels or presumed coronary vasospasm accounted for symptoms in the remaining patients. Mortality for the entire group was 0.08%. When repeat angioplasty was applied to patients with restenosis, over 80% of the initially successful group were clinically stabilized. There was a lower restenosis rate of 17% for the last 100 successful patients and this observation may be related to higher inflation pressures achieved with newer balloons. No increase mortality was noted with the higher inflation pressures.

Project Description:

In order to evaluate the incidence and causes related to the occurrence of coronary artery restenosis after successful percutaneous transluminal coronary angioplasty (PTCA), 251 consecutive patients, who underwent a successful procedure at either the National Institutes of Health or Georgetown University Medical Center, were evaluated. Follow-up coronary angiography took place in 83 of the initial group of 89 successful patients one to eleven months after successful PTCA. Restenosis occurred in 32 patients (36%), with all but two patients having either recurring angina and/or a positive exercise test. The other two patients had significant collateral flow to the vessel with restenosis. Conversely, 39 of 41 asymptomatic patients did not have restenosis. For the entire group of 251 successes with follow-up of at least 6 months (average 21 months), 54% was asymptomatic. In the symptomatic group restenosis was found in 63% of patients. Progression of disease in other vessels or presumed coronary vasospasm accounted for symptoms in the remaining patients. Mortality for the entire group was 0.08%. When repeat angioplasty was applied to patients with restenosis, over 80% of the initially successful group were clinically stabilized. There was a lower restenosis rate of 17% for the last 100 successful patients and this observation may be related to higher inflation pressures achieved with newer balloons. No increase mortality was noted with the higher inflation pressures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04079-0 1 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The natural history of mitral regurgitation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Joann Urquhart, M.D.	Sr. Medical Staff Fellow	CB NHLBI
Robert O. Bonow, M.D.	Sr. Investigator	CB NHLBI
Barry J. Maron, M.D.	Head, Echo Lab	CB NHLBI
Stephen Bacharach, Ph.D.	Physicist	NM CC
Michael V. Green, M.D.	Chief, Applied Physics Sect.	NM CC
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Dept., Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Determining the optimal time for valve replacement surgery in patients with mitral regurgitation (MR) is an important clinical problem. Patients with few or no symptoms, despite severe mitral regurgitation, present a particular problem. Since some patients remain asymptomatic for 10-20 years, it would be unwise to subject a patient prematurely to an operation which has a high operative mortality (5-10%) or the risks of a prosthetic valve. On the other hand, waiting too long can increase the surgical mortality and jeopardize the postoperative effect, resulting in irreversible left ventricular dysfunction.

This protocol was initiated by the Cardiology Branch in 1978 to study the natural history of two groups of patients with MR; those who were asymptomatic with those who were operative candidates. Invasive (cardiac catheterization) and noninvasive (M-mode echo, stress tests and radionuclide angiography) parameters of LV function were performed in an effort to find an objective predictor of clinical deterioration.

What is clear so far is by the time patients with MR become symptomatic enough to require operation, most have sustained left ventricular dysfunction which appears to be irreversible. Preoperative left ventricular dilatation by M-mode echo (LVDD>70 or LVSD>45) may identify patients at greater risk for severe postoperative LV dysfunction. The clinical significance of this depressed postoperative LV function remains to be determined by long-term follow-up studies.

It is crucial that the nonoperated and the operated patients continue to have follow-up noninvasive studies to further determine what preoperative parameters influence post-operative clinical course and to prove the relationship of these findings to the natural history of MR patients postoperatively.

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Project Description:

Previous studies have demonstrated that the LV ejection fraction (EF) of patients with mitral regurgitation deteriorates significantly following valve replacement. However, the long term clinical significance and effect on long-term LV function of this finding are unknown. We therefore studied the effects of mitral valve replacement on early postop (6 months to 2 years) and late postop (3 to 5 years, mean 3.5) LV function in 23 mitral regurgitation patients using radionuclide angiography. Before mitral valve replacement, ejection fraction was 48±8% (mean±SD); ejection fraction early after mitral valve replacement decreased to 35±13% ($p<0.001$) with 12 patients demonstrating >5% decrease in ejection fraction. The only patient who died during subsequent follow-up was the patient with the lowest ejection fraction early post-op (13%, compared to 35% preop). All remaining patients are alive; 19 of 22 have persistant improvement of ≥1 functional class compared to preop values. At 3-5 years ejection fraction was 39±14%, a significant increase ($p<.005$) from the early post-op studies; in 9 patients ejection fraction increased >5%. All patients with ejection fraction >30 on early study remained stable or showed an increase in ejection fraction on late study (43±9% to 50±10%, $p<.05$). However, of 8 patients with ejection fraction <30% on early study, 1 patient died, two had no change in ejection fraction between early and late studies, and 3 had a progressive decrease in ejection fraction of >5%. Hence, early post-op deterioration in ejection fraction does not generally correlate with subsequent clinical or LV functional course; most patients with substantial early decrease in ejection fraction manifest improved long-term LV function, with good clinical results 3-5 years postop. However, patients with profoundly decreased ejection fraction post-op (<30%) appear to constitute a group at risk of persistant LV dysfunction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201-HL-04080-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Calcium channel blocker therapy in patients with abnormal vasodilator reserve

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Snr. Medical Staff Fellow	CB	NHLBI
Rita M. Watson, M.D.	Medical Staff Fellow	CB	NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB	NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Abnormal vasodilator reserve of the coronary microcirculation has recently been demonstrated to be a frequent mechanism of angina pectoris in patients with "normal" coronary arteries. To asssess the effect of calcium antagonist drugs known to be potent coronary arteriolar vasodilators, 25 patients previously demonstrated to have angina pectoris on the basis of abnormal vasodilator reserve underwent an outpatient randomized double-blind, placebo controlled, crossover study. An unblinded lead-in phase determined the best dose of verapamil or nifedipine for each patient. The period of treatment for placebo or drug during the study was 28 days, during which time diaries were kept to record episodes of chest pain and nitroglycerin consumption. Exercise testing using bicycle ergometry was performed at the end of each treatment period. Fifteen patients received verapamil 160-640 mg/day; 10 patients received nifedipine 40-120 mg a day. Values represent mean $\pm$ SD. ETT = exercise tolerance testing.

	Episodes of Chest pain	Nitroglycerin Consumption	ETT Duration	ETT Chest pain
Placebo	35±28	40±53	4'30± 2'30"	16/21
Drug	22±22*	22±28*	5'13± 2'20"**	9/24^{+}

(*=p<0.005, **=p<0.025, $^{+}$=p<0.01 vs. respective placebo)

Four patients interrupted the study during the placebo phase; one patient interrupted the study during the drug phase. Thus calcium antagonist therapy appears beneficial in controlling anginal symptoms and improving exercise tolerance in patients with angina pectoris, "normal" coronary arteries and abnormal vasodilator reserve.

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Project description:

Abnormal vasodilator reserve of the coronary microcirculation has recently been demonstrated to be a frequent mechanism of chest pain in patients with angiographically normal-appearing coronary arteries. To assess the effect of calcium antagonist drugs known to be patent coronary arteriolar vasodilators, 25 patients previously shown to have abnormal vasodilator reserve underwent an outpatient randomized double-blind, placebo controlled study. Each patient received nifedipine or verapamil in doses previously found to be effective in controlling chest pain symptoms. The period of treatment was 28 days, during which time diaries were kept to record episodes of chest pain and nitroglycerin consumption. Exercise testing was performed at the end of each treatment period. Patients treated with calcium channel blockers experienced significantly fewer episodes of chest pain, consumed less nitroglycerin, and demonstrated improved exercise tolerance compared to placebo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04081-01 CB

PERIOD COVERED: October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Myogenic control of reactive hyperemia during coronary constriction and ischemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Mary F. Maturi, M.D.	Clinical Associate	EPPS CB NHLBI
Maret Maxwell, Ph.D.		MES BEIB DRS
Randolph E. Patterson, M.D.	Head	EPPS CB NHLBI

COOPERATING UNITS *(if any)*

Mechanical Engineering Section (MES), Biomedical Engineering and Instrumentation (BEIB), Division of Research Services (DRS)

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology Section (EPPS)

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The two major theories that attempt to explain the increase in coronary blood flow (CBF) that follows release of a brief coronary occlusion are the vasodilator metabolite and the myogenic tone theories. We hypothesized that if the heart muscle were already ischemic, a brief coronary occlusion should produce little further increase in metabolites and little or no reactive hyperemic increase in CBF. We previously found that arginine-8-vasopressin (AVP) and neuropeptide tyrosine (NPY) caused coronary vasoconstriction severe enough to produce myocardial ischemia. Thus we used the same doses of AVP (n=5) or NPY (n=6) in the same experimental model, (chloralose-anesthetized, open chest dogs) and found the same decreases in CBF. We compared the reactive hyperemic increase in CBF after release of 15 sec coronary occlusions before vs after one of the peptides had reduced CBF by 41-48%. Peak reactive hyperemic CBF was reduced by only 18-22% (p=N.S.) after peptide-induced vasoconstriction that was severe enough to produce myocardial ischemia. These results are difficult to explain by the vasodilator metabolite theory because the ischemia existing before the coronary occlusion should have stimulated production of vasodilator metabolites. If the virtually normal vasodilator reserve manifested during reactive hyperemia had been available to the heart during peptide-induced vasoconstriction, then ischemia might have been prevented. These findings suggest an additional, non-metabolic stimulus to reactive hyperemia. We measured distal coronary branch pressure (DCP) to assess indirectly the myogenic stimulus for coronary arterial tone. DCP was not altered by peptide-induced vasoconstriction, and occlusion reduced DCP to similar values before vs during peptide infusion. The same decrease in DCP during occlusion should provide the same decrease in myogenic tone as a stimulus for reactive hyperemia with or without vasoconstriction. Thus, these data support an important role for changes in myogenic tone to regulate the reactive hyperemic increase in CBF.

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Project Description:

Stenosis of a large coronary artery to cause even a mild decrease in resting coronary blood flow (CBF) abolishes reactive hyperemia(RH). We reported that transmural ischemia resulted from a 39- 50% reduction in CBF produced by intracoronary (IC) arginine-8 vasopressin(AVP) or neuropeptide tyrosine(NPY). To determine whether this peptide-induced vasoconstriction also abolished RH, 12 chloralose-anesthetized openchest dogs were studied. Distal coronary pressure(DCP) and CBF were measured during baseline(B), coronary occlusion(CO) and peak RH following release of a 15 sec CO before and after IC AVP(0.1 nmoles, n=6) or NPY(42 nmoles,n=6)

	CBF-B	CBF-RH	DCP-B	DCP-CO	DCP-RH
Control	37	149	111	23	62
AVP	22*	122	113	28	59
Control	38	133	97	17	50
NPY	20*	104	108	18	56

*$p < .001$ vs control. Remarkably, the apparent vasodilator reserve seen during RH was not available to offset vasoconstrictor-induced ischemia. Since this ischemia itself should stimulate a near maximal metabolic vasodilator response, another stimulus for RH during vasoconstriction must be considered to fully explain this degree of preserved RH. DCP dropped during CO to similar values before vs after vasoconstriction. This decreased DCP would cause similar reductions in coronary myogenic tone and, thus stimulate similar RH. Conclusion: the decrease in myogenic tone resulting from the decrease in DCP can explain more fully preserved RH during vasoconstriction severe enough to produce ischemia. These studies support an important role for myogenic tone in regulation of RH and suggest a need for cautious interpretation of RH as an index of vasodilator reserve available to prevent ischemia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04082-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Role of large vs. small coronary vessels in neuropeptide-induced ischemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name title, laboratory, and institute affiliation)*

Mary Maturi, M.D.		EPPS	CB NHLBI
Maret Maxwell, Ph.D.		MES	BEIB DRS
Randolph E. Patterson, M.D.	Head	EPPS	CB NHLBI

COOPERATING UNITS *(if any)*

Mechanical Engineering Section (MES), Biomedical Engineering (BEIB), Division of Research Services (DRS), Experimental Physiology and Pharmacology Section (EPPS)

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology Section (EPPS)

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

We found previously that two naturally occurring peptides, arginine-8-vasopressin (AVP) and neuropeptide tyrosine (NPY) both decreased coronary blood flow (CBF) by 36-41% without changing aortic blood pressure (AoP) after infusion directly into the coronary arteries of chloralose-anesthetized, open-chest dogs. Most importantly, we found that the vasoconstriction due to both AVP and NPY was severe enough to produce myocardial ischemia, as shown by impaired left ventricular contraction and development of tissue acidosis. We hypothesized that these peptides caused ischemia by constricting large coronary arteries remote from the ischemic cells which produce vasodilator-metabolites. We tested this hypothesis in 12 dogs prepared like the groups which demonstrated ischemia due to peptides. Resistances of large (R-L) and small (R-S) coronary arteries were calculated by dividing the respective pressure drops by the CBF, after a small, distal coronary branch was cannulated to measure distal coronary pressure (DCP). Intracoronary administration of AVP and NPY decreased CBF by 41-48% without changing AoP or DCP. These were the same peptide doses that had produced myocardial ischemia. R-L increased from 0.22 to 0.39 units and R-S from 2.70 to 5.29 units ($p<.05$ for R-S but not for R-L). More importantly, the change in R-S accounted for 93-94% of the change in total resistance (R-L + R-S). Thus the peptide-induced vasoconstriction was due primarily to the constriction of small coronary arteries, not the large arteries. These findings indicate the strength of vasoconstriction due to AVP and NPY because both peptides compete directly with the vasocilator metabolites near the small arteries or arterioles involved in normal regulation of CBF. We conclude that constriction of small coronary arteries can overcome the powerful processes that normally regulate small coronary arteries to control blood flow. This small vessel vasoconstriction can produce myocardial ischemia.

Project Description:

We reported that arginine-8-vasopressin (AVP) and neuropeptide tyrosine (NPY, recently discovered in human coronary arteries) overcame autoregulation by causing vasoconstriction severe enough to produce myocardial ischemia. However the site of vasoconstriction remains unknown. Thus to determine whether this vasoconstriction was due to increased resistance in large (R_L) or small (R_S) coronary arteries, we measured aortic pressure (AP), circumflex coronary blood flow (CBF), distal coronary pressure (DCP) and LV diastolic pressure (LVDP). We calculated R_L=(AP-DCP/CBF) and R_S=(DCP-LVDP/CBF) and total resistance (R_T=R_L+R_S=mmHg/ml/min) in 12 chloralose-anesthetized open-chest dogs before and after 0.1 nmoles of intracoronary (IC) AVP(n=6) or 42 nmoles IC NPY (n=6).

	AoP	R_T	R_L	R_S	R_S/ R_T
Control	108	3.02	.22	2.80	--
AVP	108	5.19*	.37	4.82*	93%
Control	102	2.83	.22	2.61	--
NPY	109	6.20*	.41	5.79*	94%

*$p<.05$ vs control. Thus R_L increased marginally ($.05<p<.1$); but R_S increased significantly and accounted for 93% to 94% of the increase in R_T. In conclusion, constriction of small coronary arteries (not seen angiographically) can produce myocardial ischemia without significant changes in large coronary arteries. Thus, both neuropeptides constrict at the level of small coronary arteries, and this degree of constriction is severe enough to override normally powerful autoregulatory mechanisms and produce ischemia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04083-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Norepinephrine interacts with neuropeptide-Y to potentiate myocardial ischemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Mary F. Maturi, M.D.	Medical Staff Fellow	EPPS CB NHLBI
Maret Maxwell, Ph.D.		MES BEIB DRS
Seth R. Goldstein, Ph.D.		MES BEIB DRS
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB NHLBI
Randolph E. Patterson, M.D.	Senior Investigator	EPPS CB NHLBI

COOPERATING UNITS *(if any)*

Mechanical Engineering Section, Biomedical Engineering and Instrumentation Branch, Division of Research Services

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology Section (EPPS)

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	0.7	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As part of a basic investigation of chemical messengers potentially involved in regulation of the coronary circulation we found previously that a peptide isolated from brain and human coronary arteries, neuropeptide tyrosine (NPY), caused coronary vasoconstriction. This 36-50% reduction in coronary blood flow (CBF) with no change in aortic blood pressure (AoP) produced myocardial ischemia, as manifested by left ventricular contractile dysfunction and myocardial tissue acidosis. Neuromodulation represents an important new concept of neural function whereby one compound modifies the action of a neurotransmitter, and it has been suggested that norepinephrine (NE) and NPY may interact in this way in sympathetic nerve endings. We tested whether there was an interaction between these compounds by infusing NPY and NE, (to increase AoP by 20 mmHg), individually and combined, directly into the coronary artery of 12 chloralose- anesthetized, open-chest dogs. We measured CBF, AoP, and as an index of myocardial ischemic injury, intramyocardial pH, by a special fiber optic probe. NE alone produced no change in pH despite a 78% increase in the product of AoP times heart rate (AoP X HR), an index of myocardial oxygen demand. Ischemia was prevented by a 135% increase in CBF, as expected. NPY alone, on the other hand decreased intramyocardial pH by 0.07 units ($p<0.05$); this was associated with no change in AoP x HR but a 39% decrease in CBF, to confirm our previous results. NE combined with NPY produced more severe acidosis than NPY alone: intramyocardial pH decreased by 0.13 vs 0.07 ($p<0.05$). The increase in AoP x HR was identical to the increase with NE alone (78%), but CBF increased less than with NE alone (76 vs 135%; $p<0.05$). We conclude that NE combined with NPY produced myocardial ischemia, in contrast to NE alone, by restricting the ability of the coronary arteries to deliver sufficient CBF to meet increased myocardial oxygen demands. Thus, an interaction exists between NE and NPY and potentiates the production of myocardial ischemia by NPY.

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Project Description:

Neuropeptide tyrosine(NPY), a possible neurotransmitter isolated from brain and human coronary arteries, was reported by us to cause vasoconstriction severe enough to produce myocardial ischemia. To determine whether an interaction exists between the effects of neuropeptide tyrosine and norepinephrine(NE) we measured circumflex blood flow (CBF), aortic pressure(AP), and heart rate(HR) in 12 chloralose-anesthetized open-chest dogs. In Group(Gr)I (n=5) NE was infused intracoronary(IC) 10 ug/min to a 20mmHg increase in AP, and in GrII(n=7) NE was infused IC at similar doses 10 to 20 min after IC NPY (42 nmoles). As an index of ischemia, intramyocardial pH was measured in infused regions(IR) and noninfused regions (NR).
Table=change in pH and % change CBF and APxHR.

	CBF	APxHR	PH-IR	pH-NR
GrI NE alone	+135*@	+78*@	-.01@	-.01
GrII NPY alone	-39*Ø	-4Ø	-.07*Ø	.00
NPY+NE	+76*@Ø	+78*@Ø	-.13*@Ø	-.01

*p<.05 vs control, @p<.05 vs NPY alone, Øp<.05 vs NE alone.

1) Norepinephrine alone did not produce ischemia, 2) while neuropeptide tyrosine alone did. 3) Norepinephrine after neuropeptide tyrosine potentiated myocardial ischemia, compared to neuropeptide tyrosine alone, associated with increases in oxygen demand and circumflex blood flow. 4) Norepinephrine after neuropeptide tyrosine caused ischemia, in contrast to norepinephrine alone, by limiting the increase in circumflex blood flow. Conclusion: neuropeptide tyrosine attenuates the increase in circumflex blood flow caused by norepinephrine; an interaction exists between norepinephrine and neuropeptide tyrosine and potentiates the production of myocardial ischemia compared to either compound alone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04084-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Coronary vasoconstriction by a thromboxane mimic: drug-induced potentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Dennis L. Sprecher, M.D.		MDB NHLBI
Joseph S. Pierce, D.V.M.		SB NHLBI
Stephen E. Epstein, M.D.	Chief	CB NHLBI
Randolph E. Patterson, M.D.	Head	EPPS CB NHLBI

COOPERATING UNITS *(if any)*

Molecular Disease Branch (MDB), NHLBI, Surgery Branch (SB) NHLBI, Experimental Physiology and Pharmacology Section (EPPS), Cardiology Branch

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology Section (EPPS)

INSTITUTE AND LOCATION

NHLBI NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Thromboxane is released from blood vessel walls and causes coronary vasoconstriction, but there is evidence that thromboxane alone cannot explain coronary spasm in man. Since thromboxane is unstable, we used its stable mimic, U-46619 (Txm) to test whether Txm interacts with other vasoactive drugs or endogenous hormones. We gave graded doses of Txm, intracoronary (IC) to 10 anesthetized, open-chest pigs before and after the combination of arginine-8-vasopressin (AVP) and ergonovine (Erg), IC. Txm caused a dose-related increase in coronary vascular resistance (CVR = mean aortic pressure/mean coronary blood flow = mm.Hg/ml/min), without changing heart rate or aortic pressure. AVP + Erg increased CVR during the baseline before the second set of Txm doses: 3.29 ± 1.27 to 4.06 ± 1.20 mm.Hg/ml/min, ($p<.05$). The second set of Txm doses after AVP Erg showed greater relative and absolute increases in CVR than did the first set of Txm doses ($p=.05$).

	% Increase in CVR after Txm dose (ug) * mean±SD%		
Txm dose	0.1	0.5	1.0
Before	*13±10%	23±21%	47±35%
After AVP Erg	23±12%	43±13%	110±93%

The effect of AVP + Erg on the vasoconstrictor response to Txm was greater than their effect on the "control" CVR before Txm ($p<.01$). Analysis of variance confirmed a true interaction rather than a simple additive effect of Txm and the combination of AVP and Erg ($p<.050$). In conclusion, these data define a synergistic effect between Txm and AVP + Erg and illustrate the potential for a multifactorial etiology of coronary vasoconstriction. This type interaction may be relevant to other vasoconstrictors. The mechanism of this interaction is not known. It is unlikely that these results are explained mechanically by a similar strength of Txm-vasoconstrictor stimulus superimposed on a vascular bed already constricted by AVP + Erg because of the greater percent increase in CVR with Txm after AVP + Erg.

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Project Description:

Although thromboxane and its stable mimic (Txm, U-46618) act as coronary vasoconstrictors, it is not known whether Txm interacts with other vasoconstrictor agents. Thus, we measured dose-response curves to intracoronary (IC) Txm in 10 anesthetized, open-chest adult pigs before and 6 min after ergonovine (E, .2mg, IC) and arginine-8-vasopressin (AVP, .01 mg, IC). Txm IC did not change heart rate or mean aortic pressure (AP), and Txm caused brief, reproducible increases in coronary vascular resistance (CVR = AP/coronary blood flow). E + AVP increased "control" CVR before the second series of Txm doses (3.29 ± 1.27 vs 4.06 ± 1.20 mm Hg/ml/min, $p<.05$), but both absolute and relative increases in CVR due to Txm were greater after E + AVP ($p=.05$) by covariance).

	% Increase in CVR after Txm Dose (mg)* mean + SD %		
Txm dose	0.1	0.5	1.0
Before	*13±10%	23±21%	47±35%
After E + AVP	23±12%	43±13%	110±93%

The effect of E + AVP on the vasoconstrictor response to Txm was greater than their effect on the "control" CVR before Txm ($p<.01$). ANOVA confirmed a true interaction rather than a simple additive effect of Txm and E+AVP ($p<.05$). Conclusions: 1) a greater % CVR increase with Txm after E+AVP makes it unlikely that these data are explained mechanically by a similar % CVR increase superimposed on a vascular bed already constricted by E + AVP; 2) these data define a synergistic effect between Txm and E + AVP and illustrate the potential for a multifactorial etiology of coronary vasoconstriction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04085-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Survival after Valve Replacement for Aortic Regurgitation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R.O. Bonow	Senior Investigator	CB, NHLBI
A.L. Picone	Medical Staff Fellow	SB, NHLBI
D.R. Rosing	Senior Investigator	CB, NHLBI
C.L. McIntosh	Senior Investigator	SB, NHLBI
M. Jones	Senior Investigator	SB, NHLBI
S.L. Bacharach		NM, CC
M.V. Green	Chief, Imaging Physics Section	NM, CC
S.E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*

Cardiac Surgery Branch, NHLBI
Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6	6	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We studied the effect of pre-operative left ventricular function on prognosis after aortic valve replacement for aortic regurgitation. We performed echocardiography and radionuclide angiography on 94 consecutive patients undergoing operation from 1976 to 1983. For all patients, the five year survival was 84 ± 5%, significantly better than our results for 1972-1976. Pre-operative resting left ventricular ejection fraction and fractional shortening were the most significant predictors of survival ($p < .001$ by univariate life table analysis). 5 year survival was 71 ± 9% in patients with subnormal ejection fraction compared to 95 ± 3% with normal ejection fraction. By multivariate analysis, left ventricular diastolic and systolic dimensions were also significant predictors of postoperative survival ($p < .01$). Survival was not influenced by the type of intra-operative myocardial preservation, nor did the type of myocardial preservation alter the influence of left ventricular function on prognosis. Hence, despite improved operative techniques and better long term survival compared to earlier results, preoperative resting left ventricular dysfunction continues to identify patients with aortic regurgitation at risk of death after aortic valve replacement.

Project Description

Objectives:

The current research of this laboratory is the following:

Recent studies suggest that left ventricular function may no longer be an important determinant of survival after operation for aortic regurgitation because of improved operative techniques. To assess the effect of left ventricular function on prognosis in the current surgical era, we performed echocardiography and radionuclide angiography on 94 consecutive patients undergoing aortic valve replacement from 1976-1983. Cold hyperkalemic cardioplegia was used for preservation in 49 patients. For all patients, 5 year survival was 84 ± 5%, significantly better than our results for 1972-1976. Preoperative rest left ventricular ejection fraction and fractional shortening were the most significant predictors of survival ($p < .001$ by univariate life table analysis): 5 year survival was 71 ± 9% in patients with subnormal ejection fraction ($n = 45$) compared to 95 ± 3% with normal ejection fraction. Patients with subnormal left ventricular ejection fraction and poor exercise tolerance or prolonged duration of left ventricular dysfunction (> 2 years) comprised the high risk group (5 year survival 61%). By multivariate analysis, left ventricular diastolic and systolic dimensions were also significant predictors of postoperative survival ($p < .01$). Survival was not influenced by hyperkalemic cardioplegia and hyperkalemic cardioplegia did not alter the influence of left ventricular function on prognosis. Hence, despite improved operative techniques and better long term survival compared to earlier results, preoperative resting left ventricular dysfunction continues to identify patients with aortic regurgitation at risk of death after aortic valve replacement. Early operation in such patients may result in further improvement in survival.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04086-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regional Left Ventricular Function in Hypertrophic Cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4	4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We investigated in the influence of regional left ventricular asynchrony on impaired global left ventricular diastolic filling in patients with hypertrophic cardiomyopathy using radionuclide angiography. Previous studies indicate that left ventricular diastolic filling is impaired in hypertrophic cardiomyopathy and is improved during verapamil therapy. We subdivided the left ventricular region of interest on the radionuclide angiogram into 20 sectors and computed the time interval from the R wave to minimum volume of each sector's time activity curve. The inter-sector standard deviation of time minimum volume (SDTMV) was used as an index of relative left ventricular regional asynchrony. This index was increased in hypertrophic cardiomyopathy patients compared to normal, indicating asynchronous regional function. After 1-2 weeks of verapamil, SDTMV significantly decreased, indicated more synchronous LV regional behavior, specifically greater systolic temporal homogeneity. This improvement after verapamil was associated with no change in global LV ejection fraction or peak ejection rate; however, global rapid-diastolic filling improved, manifested by an increased peak filling rate. These data suggest that in many hypertrophic cardiomyopathy patients, impaired global left ventricular filling may be associated, in part, with non-uniformity of systolic function and that verapamil's beneficial effects on left ventricular diastolic function may be mediated by reduction in regional asynchrony.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description

Objectives:

The current research of this laboratory is the following:

Left ventricular diastolic filling is impaired in hypertrophic cardiomyopathy. To assess the influence of left ventricular regional asynchrony on global left ventricular filling, we studied 48 hypertrohic cardiomyopathy patients by radionuclide angiography before and during oral verapamil therapy. Left ventricular regional synchrony was assessed by dividing the left ventricular region of interest into 20 sectors and computing the time interval from the R wave to minimum volume of each sector's time activity curve. The inter-sector standard deviation of time to minimum volume (SDTMV) was used as an index of left ventricular regional temporal homogeneity. SDTMV was increased in hypertrophic cardiomyopathy patients compared to normal (34 ± 15 ml vs 20 ± 7 ms, $p < .001$) indicating asynchronous regional function. After 1-2 weeks of verapamil, global left ventricular ejection fraction and peak ejection rate were unchanged, but global rapid diastolic filling improved: peak filling rate increased (3.3 ± 1.1 to 4.3 ± 1.3 end diastolic volume/sec, $p < .001$) and time to peak filling rate decreased (189 ± 43 to 170 ± 29 ms, $p < .005$). These changes were associated with a reduction in SDTMV (from 34 ± 15 ms to 24 ± 10 ms, $p < .001$). Hence, improved global left ventricular filling after verapamil was associated with more synchronous left ventricular regional behavior, specifically greater systolic temporal homogeneity. These data suggest that in many hypertrophic cardiomyopathy patients, impaired global left ventricular filling may result, in part, from non-uniformity of systolic function and that verapamil's beneficial effects on left ventricular diastolic function may be mediated by reduction in asynchrony.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04087-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Verapamil Effect on Exercise Tolerance and LV Filling in Hypertrophic Cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)*

PI: R.O. Bonow	Senior Investigator	CB, NHLBI
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LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4	4	0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Diastolic filling of the left ventricular is impaired in many patients with hypertrophic cardiomyopathy. Although verapamil improves indices of diastolic filling in many patients with this disease, the relation between improved diastolic filling and improved exercise tolerance has not been demonstrated. To investigate the association between changes in left ventricular filling and exercise tolerance after verapamil, we studied 43 patients by radionuclide angiography and graded treadmill testing before and after 1-4 weeks of oral verapamil therapy, 320-480 mg per day. The verapamil-induced increase in peak left ventricular filling rate (PFR) at rest was associated with a increased exercise tolerance: exercise capacity increased in 28 of 32 patients manifesting an increase in PFR but only 3 of 11 patients with unchanged or decrease PFR. This initial trend persisted in 23 patients studied after 1 year of therapy: 11 of 14 patients with a persistent increase in PFR had persistent improvement in exercise tolerance relative to pre-verapamil values, compared to only 1 of 9 patients in whom PFR was unchanged or decreased relative to pre-verapamil values. Hence, verapamil-induced changes in left ventricular PFR were associated significantly with objective symptomatic improvement. These data indicate that enhanced left ventricular diastolic filling is an important mechanism contributing to the clinial improvement experienced by many hypertrophic cardiomyopathy patients during verapamil therapy.

Project description

Objectives:

The current research of ths laboratory is the following:

Verapamil improves exercise tolerance and decreases symptoms in many patients with hypertrophic cardiomyopathy. The mechanisms responsible for these effects are not understood completely, although previous studies indicate that verapamil enhances left ventricular relaxation and diastolic filling in such patients. To investigate the association between changes in left ventricular filling and exercise tolerance after verapamil, we studied 43 hypertrophic caridomyopathy patients by radionuclide angiography and graded treadmill testing before and after 1-4 weeks of oral verapamil therapy, 320-480 mg per day. The verapamil-induced increase in peak left ventricular filling rate at rest (from 3.0 ± 1.2 to 3.6 ± 1.1 end-diastolic volume/sec, $p < .001$) was associated with an increase in exercise tolerance (from 5.9 ± 3.9 to 9.0 ± 5.0 min, $p < .001$): exercise capacity increased in 28 of 32 patients manifesting an increase in peak filling rate but only 3 of 11 patients with unchanged or decrease peak filling rate ($p < .001$). This initial trend persisted in 23 patients studied after 1 year of therapy: 11 of 14 patients (79%) with a persistent increase in peak filling rate had persistent improvement in exercise tolerance relative to pre-verapamil values, compared to only 1 of 9 patients (11%) in whom peak filling rate was unchanged or decreased relative to pre-verapamil ($p < .01$). The short and long-term changes in peak filling rate also correlated with changes in functional class ($p < .001$). Hence, verapamil-induced changes in left ventricular peak filling rate were associated significantly with subjective and objective symptomatic improvement. These data indicate that enhanced left ventricular diastolic filling is an important mechanism contributing to the clinical improvement experinced by many hypertrophic cardiomyopathy patients during verapamil therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04088-01 CB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Dynamic Pressure-Volume Relations during Ejection in Hypertrophic Cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)*

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M.B. Leon		CB, NHLBI
R.M. Watson		CB, NHLBI
M.V. Green	Chief,	NM, CC
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COOPERATING UNITS *(if any)*

Nuclear Medicine, Clinical Center

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SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

We studied the dynamics of left ventricular outflow tract gradients in hypertrophic cardiomyopathy. In 25 patients, we performed catherization with micromanometer catheters and a nonimaging scintillation probe to construct high temporal resolution left ventricular pressure-volume loops. In 11 patients without outflow tract gradients, the contour of the ejection phase was concave downward. In the 14 patients with outflow tract gradients, however, an inflection point occurred during the ejection phase, corresponding to onset of the left ventricular outflow tract gradient. After this inflection point, left ventricular pressure increased markedly, and the difference between peak left ventricular pressure and this inflection point correlated with the magnitude of the outflow tract gradient. This point appeared early during ejection and the mean percentage of left ventricular stroke volume that occurred after this point and during the outflow tract gradient was 62%. These data indicate that the outflow tract gradient in hypertrophic cardiomyopathy cannot represent cavity obliteration after left ventricular emptying, as over half of left ventricular stroke volume is ejected during the outflow tract gradient. These data provide further evidence that the outflow tract gradient represents true impedence to left ventricular ejection.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description

Objectives:

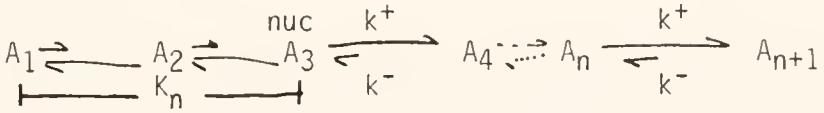
The current research of this laboratory is the following:

Whether the left ventricular outflow tract gradient represents obstruction to left ventricular ejection in hypertrophic cardiomyopathy remains controversial. To assess the dynamics of the outflow tract gradient, we studied 25 hypertrophic cardiomyopathy patients at catheterization with micromanometer catheters and a nonimaging scintillation probe to construct high temporal resolution left ventricular pressure-volume loops. In 11 patients without outflow tract gradients, the contour of the ejection phase was concave downward. In the 14 patients with outflow tract gradients, however, an inflection point (PVi) occurred during the ejection phase, corresponding to onset of the left ventricular outflow tract gradient. Left ventricular pressure increased markedly after PVi, and the difference between peak left ventricular pressure and the pressure at PVi correlated with the magnitude of outflow tract gradient ($r = 0.95$). PVi appeared early during ejection (100-150 ms after the R wave), and 62 ± 7% (range 49-73%) of left ventricular stroke volume occurred after PVi and during the outflow tract gradient. These data indicate that the outflow tract gradient cannot represent cavity obliteration after left ventricular emptying, as over half of left ventricular stroke volume ensures during the outflow tract gradient, and provide further evidence that the outflow tract gradient represents true impedence to left ventricular ejection.

Annual Report of the Labortory of Cell Biology,
National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

Actin Polymerizaton: In this continuing project, Dr. Korn and his collaborators are studying the mechanism and regulation of the polymerization of actin, a major cytoskeletal and contractile protein in all eukaryotic cells. Understanding the polymerization process is very important because, especially in nonmuscle cells, polymerization and depolymerization are continuous processes through which cell shape, organization and motile activity are regulated. Our major effort this year was to understand the role of ATP hydrolysis in the polymerization process.

Actin polymerization has long been described as a slow nucleation process followed by a rapid elongation step and then a slow rearrangement of the filament number/length distribution after polymerization is complete. Last year, we showed that, under a variety of conditions, the data for the first two steps are best described by a model in which the nucleus is an actin trimer that is in unfavorable pre-steady state equilibrium with actin trimer that is in unfavorable pre-steady state equilibrium with monomer. Once formed, trimer elongates to polymeric F-actin.



This system can be described by the pre-steady state equilibrium constant, K_n, for nucleus formation and association and dissociation rate constants, k^+ and k^-, for elongation.

This year we found that the same general polymerization scheme fits the data for polymerization of ADP·actin as for ATP·actin. Furthermore, by nucleating polymeriztaion with a known concentration of covalently crosslinked actin trimers, it was possible to obtain absolute values for the rate constants for polymerization in ATP and ADP and for the critical concentration, C_c, under both conditions. The C_c is the concentration of monomer in equilibrium (ADP) or steady state (ATP) with polymer. The C_c in ADP is about 30 times greater than in ATP because k^+ is lower and k^- is larger in ADP than in ATP. This establishes one of the important consequences of the hydrolysis of ATP in the polymerization reaction. The actin polymer is much more stable in the presence of ATP than in ADP.

A number of experimental approaches have demonstrated that, at steady state in ATP, the ends of the actin filaments have one or more subunits with bound ATP while the bulk of the polymer contains ADP·actin subunits. The ATP cap occurs because polymerization and ATP hydrolysis are not mechanistically coupled events. Hydrolysis of ATP occurs on the filament after (and more slowly than) the addition of the ATP·actin subunit. It is this ATP cap that stabilizes the actin filament. In fact, it is the continuous hydrolysis of ATP at steady state that stabilizes the filament, not just the existence of the ATP cap. We have demonstrated that under conditions where ATP hydrolysis does not occur, and the polymer contains only ATP·actin subunits, the F-actin is less stable. Thus, both the equilibrium polymer containing ADP and the one containing ATP subunits are less stable, i.e. have higher critical concentrations, than the

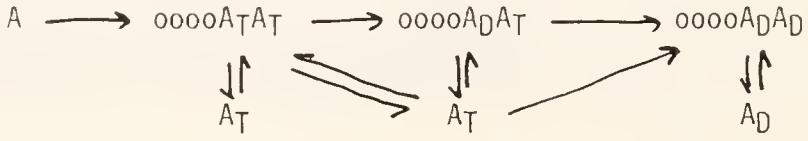
steady state polymer that occurs when hydrolysis of ATP accompanies the polymerization reaction.

Conditions can be established, however, in which, even though ATP is present there is no ATP cap. This is done by greatly increasing the number of filament ends by sonication which exposes internal ADP·subunits which will then dissociate at the rate dictated by the k^- for ADP·actin. The exchange of ATP for the ADP on the actin is slower than the dissociation of actin subunits so that ADP remains bound to the filament ends and also the concentration of ADP·monomer accumulates so that the C_c approaches the higher value for ADP·actin, even though excess ATP is present. Therefore, filaments depolymerize.

Predictably, a similar situation will occur at normal steady state because a statistical fraction of filaments in ATP will lose their ATP cap (by hydrolysis of the ATP) and these filaments will rapidly depolymerize while, to maintain the steady state, ATP-capped filaments will elongate. Thus, the situation at steady state is not just a simple monomer-polymer exchange but a much more dynamic one involving large losses of subunits from some filaments and equivalent growth of others.

Finally, we have established that the filament redistribution process that occurs after polymerization is complete is not primarily due to annealing of one filament with another, as previously proposed, but is due to the complete loss of some filaments while others grow. This can be most easily understood by considering the fact, although dissociation and association events occur equally at steady state, on any one filament a series of dissociation events can occur sequentially followed by a series of association events. When this happens on a short filament, it will disappear entirely.

Thus, we can now write a much more complete scheme for the polymerization of ATP·actin which includes the consequences of ATP hydrolysis.



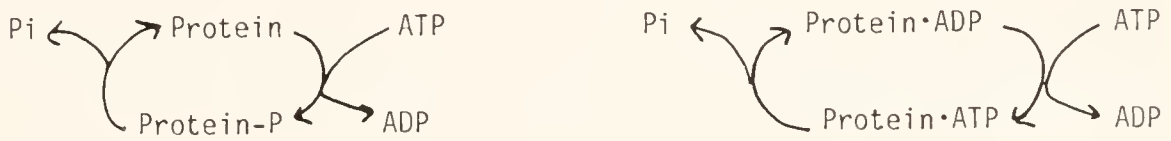
C_c = 3 μM "0.3 μM" 8 μM

According to this scheme, actin monomers (A) polymerize to form polymers with multiple ATP·actin subunits at the ends. Most of the ATP is hydrolyzed on the polymer until an ATP cap is left. But is some cases, even the ATP cap will by hydrolyzed. Thus, at steady state in ATP a number of reactions occur at the ends of filaments with a net C_c of about 0.3 μM (in 1 mM $MgCl_2$). This is not the C_c for a single reaction but is the monomer concentration for reactions in which A_T adds to A_D-filament ends and A_T-filament ends with different nucleotides at the penultimate position. In both the pure ATP situation and the pure

/15

ADP situation the C_c's are higher.

The conclusions is that the hydrolysis of ATP regulates the system by determining whether ATP or ADP will be bound to the actin with the properties of the actin depending on the nature of the bound nucleotide. The energy of hydrolysis of ATP is used in just the same way as it is in other systems in which a protein is phosphorylated and dephosphorylated through a kinase/phosphatase cycle. Non-covalent and covalent modification of proteins, therefore, are alternate ways of providing for kinetic regulation through the steady state hydrolysis of ATP.



Another major activity of Dr. Korn's laboratory is the study of the regulation of myosins of non-muscle cells through phosphorylation. Most of the recent work has been on three myosin isoenzymes from Acanthamoeba castellanii, the only non-muscle cell in which myosin isoenzymes have been detected. Definitive evidence has now been obtained that the heavy chains of the three myosins (IA, IB and II) are different gene products. Translation of Acanthamoeba mRNA by a rabbit reticulocyte system in the presence of radioactive methionine and precipitation of the translates with specific antibodies demonstrated that all three heavy chains were synthesized. Efforts to clone the myosin genes have been quite productive. At least one myosin II heavy chain gene has definitely been cloned and there is strong evidence that a myosin IB heavy chain has also been obtained. Pursuing this work should, in the immediate future, allow us to determine the sequences of these heavy chains - which will be the first non-muscle myosins sequenced - and, eventually, to produce altered heavy chains, synthetic peptides and specific antibodies to these peptides so that a number of structural-functional relationships can be pursued.

Last year, we reported that the actin-activated ATPase of myosin II was regulated by phosphorylation of three serine residues at the COOH-end of each of the two heavy chains. Controlled chymotrypsin cleavage releases a small peptide of 60-70 amino acids that contains all three phosphorylation sites. This year we have sequenced the first 58 residues from the NH_2-end of the peptide. The first 36 residues are in a sequence predicting an α-helical coiled coil structure which is then interrupted at residues 38-40. The three phosphorylatable serines occur at positions 46, 51 and 56 in nearly identical pentapeptides: -Arg-Gly-Gly-Ser-Thr-Arg-Gly-Ala-Ser-Ala-Arg-Gly-Ala-Ser-Val-Arg-. This sequence is quite different from that of the substrates for cAMP-dependent kinase and myosin light chain kinase.

The chymotrypsin-cleaved myosin II missing the above peptide has no actin-activatable ATPase activity nor is it able to make bipolar filaments. It does, however, make monopolar parallel dimers. This is of considerable interest because parallel dimers are thought to be intermediates in the formation of bipolar filaments from myosin monomers in other systems. More-

over, the dimers of chymotrypsin-cleaved myosin II bind to F-actin in the absence of ATP about as well as the intact molecule but cannot bind in the presence of ATP. Thus, the small peptide removed from the tip of the tail seems to be necessary for filament formation, for binding of myosin II to F-actin and for actin-activated ATPase activity. This agrees with our previous evidence that myosin II activity occurs and is regulated at the filament level.

Additional evidence for this hypothesis came from the study of trypsin-cleaved myosin II. Trypsin makes a single nick in the head region of the molecule but the two peptides thus formed do not dissociate except under denaturing conditions. The trypsin-cleaved myosin II still makes bipolar filaments but these filaments no longer have actin-activated ATPase activity although, as was also true for the chymotrypsin-cleaved enzyme, it retains complete Ca-ATPase activity. Also, trypsin-cleaved myosin II inactivates native, dephosphorylated myosin II when they are present in the same copolymer. This, and other, evidence suggests that the trypsin-cleaved myosin II, as also was true for phosphorylated myosin II, is unable to make filaments of the proper conformation for actin-activated ATPase activity to be expressed.

The location of the catalytic and regulatory phosphorylation sites on the myosin IA and IB heavy chains have been mapped. In both cases, the two sites are within 40 kDa of each other (the heavy chain of IA is 130 kDA and IB is 125 kDa) with the catalytic site being nearer to the NH_2-terminus.

Both myosin IA and IB are single-headed molecules that appear to be unable to make bipolar filaments. Thus, their roles in movement may be different than that of the typical myosins that do form bipolar filaments, and therefore, can function in the classical sliding filament model. That they can function in movement was established this year by demonstrating that beads coated with myosin IA or myosin IB move along cables of actin filaments. Movement required that the myosins be phosphorylated (their enzymatically active state) and was inhibited by antibodies that inhibit ATPase activity.

We have also observed that the specific activity of the actin-activated ATPase of myosins IA and IB shows actin-concentration dependent myosin cooperativity. Also, myosin IA and IB appear to be able to crosslink filaments of F-actin as demonstrated by a large increase in light scattering and a large increase in low-shear viscosity when the myosins are added to solutions of F-actin. These changes are as great as obtained with two-headed heavy meromyosin and very much greater than were seen with single-headed subfragment-1 prepared from muscle myosin. Thus, there is now indirect evidence that, in the presence of F-actin, either the myosin I isoenzymes can form oligomers or that they have two actin-binding sites. By either mechanism, myosins IA and IB could function by mechanism analogous to sliding filaments.

Biochemistry of muscle contraction: Under the direction of Dr. Evan Eisenberg and Dr. Lois E. Greene, studies on the mechanism and regulation of the actomyosin ATPase and the transduction of the energy released by ATP hydrolysis into muscle contraction continue with high productivity. Last year we discussed the evidence that led to the proposal of the following model for the hydrolysis of ATP by actomyosin in vitro:

$$M\cdot ATP \rightleftharpoons M\cdot ADP\cdot Pi_{I} \rightleftharpoons M\cdot ADP\cdot Pi_{II}$$

$$AM \rightleftharpoons AM\cdot ATP \rightleftharpoons AM\cdot ADP\cdot Pi_{I} \underset{\text{rate limiting}}{\rightleftharpoons} AM\cdot ADP\cdot Pi_{II} \xrightarrow[\text{rapid}]{} AM\cdot ADP + Pi$$

At every stage, the actomyosin (AM) and myosin (M) complexes are thought to be in rapid equilibrium and the hydrolysis reaction, ATP ADP·Pi, and the conformationl change, $ADP\cdot Pi^{I}$ $ADP\cdot Pi^{II}$, is believed to occur at the same rate on M and on AM. The difference is that the last step, product release, occurs very rapidly from AM and very slowly from M. The rate-determining step step is proposed to be the conformation step.

The usefulness of the model for the ATPase cycle is the extent to which it explains contractility. One test of the validity of this in vitro enzymatic model for the physiological event of muscle contraction is to compare the V_{max} for ATP hydrolysis with the V_{max} for muscle contraction. This was accomplished this year.

To obtain the V_{max} for the hydrolysis of ATP, muscle myosin subfragment-1 was covalently coupled to F-actin (to ensure that all of the myosin was always bound to the actin). To determine the V_{max} of muscle contraction, the rate of force redevelopment was measured after rapidly releasing and then restretching a muscle fiber. Measurements were made between 5° and 35° C and over a three-fold range of ionic strength (for muscle) and 10-fold range (for ATPase). The two values consistently agreed within a factor of 2. These results lend strong support for the above model and for its applicability to the physiological situation.

Microtubule Structure and Function: Microtubules, a major cytoskeletal structural element, consist of the polymerized tubulin dimer and several microtubule-associated proteins of which MAP-2 is the predominant species in vertebrate brain. Tubulin undergoes a post-translational tyrosinolation and MAP-2 is post-translationally modified by phosphorylation. Both these enzymatic processes are under study by a group under the leadership of Dr. Martin Flavin. This year, primary emphasis was on the phosphorylation of MAP-2.

Last year we reported that there were about 10 mol of phosphate per mol of protein on highly purified MAP-2 (A sites) and that an additional 10-12 sites (B sites) could be phosphorylated by an endogenous cAMP-dependent protein kinase that is tightly bound to the purified MAP-2. The A sites have now been labeled in vivo by injection of ^{32}Pi and the B sites labeled in vitro by incubation by incubation of the purified MAP-2 with [$-^{32}P$]ATP. Incubation with 5 different protein phosphatases removed the ^{32}P only from the B sites. This, and other, evidence strongly suggests that the A sites and B sites are functionally different sites and not just different extents of labeling of the same set of sites. By analysis of the phosphoamino acids

recovered following partial acid hydrolysis, it was determined that the A sites consist of serine and threonine residues in a ratio of 6:1.

Last year, we reported the purification from brain of a non-specific protein phosphatase that would remove B site phosphates from MAP-2 but was more active with phosphohistone as substrate. With B-site-labeled MAP-2 as substrate, a protein phosphatase has now been partially purified that has appreciable specificity for MAP-2. The crude brain extract exhibits a ratio of activity with MAP-2 and phosphohistone of about 3 but the purified enzyme has an activity ratio of about 20. With very high concentrations of the partially purified enzyme, A-site phosphates can also be released.

These studies will be extended next year to include peptide maps and other kinds of evidence to establish unequivocally that the A and B sites are distinct and non-overlapping and to compare the physiological properties (promotion of microtubule assembly) of MAP-2 with the four possible combinations of phosphorylated or non-phosphorylated A and B sites.

Membrane Recycling: In eukaryotic cells there is a rapid and extensive movement of membrane from the cell surface to internal vesicles and back to the cell surface during a number of endocytic and exocytic events. These processes are being studied under the immediate direction of Dr. Blair Bowers using the amoeba, Acanthamoeba castellanii, as a model system because it is extraordinarily active endocytically.

One measure of the rate and mechanism of recylcing of membrane is the rate of release into the medium of the hydrolases that are contained within lysosomal vesicles. Release occurs when the vesicle membrane fuses with the plasma membrane of the cell. Last year we reported that the hydrolases fall into two classes: one class is secreted at a rate of about 4% per hour and the other at about 17% per hour. We postulated that the hydrolases secreted at the lower rate were in solution in the lysosomal space while the second group were concentrated by adsorption to the membrane at the acidic pH of the vesicle interior. Then, when the vesicle membrane fused with the plasma membrane, the luminal surface of the vesicle membrane would be exposed to the higher pH of the medium and these concentrated enzymes would be released.

To test this hypothesis, the vesicle pH was raised from the normal value of about 4.8 to 6.8 by addition to the medium of either 10 mM ammonium acetate of 1 mM chloroquine. The first class of hydrolases continued to be secreted at 3% per hour while the rate of secretion of the second class was reduced from 17% to 4% per hour, as predicted. This model also explains why both classes of hydrolases are secreted at the same rate from phagocytosing (in contrast to pinocytosing) cells; the phagosomal pH is 6, a value that is probably high enough to prevent concentrative binding of enzymes to the luminal membrane surface.

The composition of plasma membranes (85-90% pure) and phagolysosomal membranes (90-95% pure) have been compared. ^{31}P-NMR spectroscopy demonstrated that the ratio of phospholipids to phosphonoglycolipids was the same in both membranes but Coomassie blue-stained electrophoretic gels revealed that only about one-third of the proteins were the same in the two membranes. Actin was a major component of the plasma membrane preparation but was not present in the phagolysosomal membranes. By ^{125}I-labeling of the outer surface

of intact cells, 8 cell surface proteins were identified. All 8 of these proteins were present in both membrane preparations so these do not account for the difference in their protein compositions. Efforts will continue to define the origin of the different protein compositions and the function of the proteins that seem to be specific to the individual membranes.

Bioenergetics: Two major problems are being investigated under the direct leadership of Dr. Richard W. Hendler. In one, new methods have been developed to identify, ultimately, all of the components of the respiratory chains of E. coli and mitochondria membranes and then to characterize them with respect to their redox potentials and the numbers of electrons passed by each. In a related study, the mechanism by which the energy generated by the passage of electrons down the respiratory chain is transduced to ATP, the form in which the energy is used by the cell, is under investigation.

To undertake the first project, new methods have been developed to collect and analyze complete absorption spectra while doing a computer-controlled potentiometric titration. Individual components of the respiratory chain are identified by their characteristic spectra and the spectrum of each component is different in its oxidized and reduced forms. Previous methods of analysis were limited to data obtained at only two specific wavelengths which may be inadequate to eliminate background light scattering and to resolve components with very similar properties. A great advantage is gained by methods that allow multiple point (as many as 200) analysis.

The major finding this year is that cytochrome c_1 of beef heart mitochondria, which was previously thought to be a single species with an Em of 230 mV capable of transferring only one electron, can be resolved into two thermodynamic species with Em values of 200 and 255 mV each of which passes two electrons at a time. Preliminary evidence suggests that cytochrome aa3, previously thought to consist of two species each passing one electron, may consist of three species which pass 2, 2 and 1 electron at a time.

It is generally agreed that the first step in energy transduction involves the development of a H^+ gradient across the membrane that contains the respiratory chain. This occurs by the translocation of H^+ at specific steps. In the oxidation of succinate, some investigators believe that the H^+/O ratio is 4, some 6 and others 8 (2 electrons are passed for every O atom consumed). All agree that a total of 4 H^+ are transported at site II and the disagreement in the literature is whether 0, 2 or 4 H^+ are transported at site III.

The previous methodology used to determine the H^+/O ratio has been difficult for several reasons: (a) generally the respiratory reaction is initiated by the addition of O_2 to an anaerobic system and there is a significant time required for mixing; (b) the electrodes have a relatively long relaxation time; (c) the electronic noise is sufficient to introduce large errors in the measurements of small voltages. As a result, the earliest measurements are recorded only after a delay of 0.6 to 1 seconds by which time an appreciable pH gradient has already developed.

New methods developed in this laboratory have allowed measurements to be made at intervals of 0.01 seconds following initiation of the reaction. The

electrodes have been greatly modified and the mixing problem has been eliminated. The mitochondria are maintained in the presence of O_2 in the dark and respiratiaon is blocked by saturating the system with carbon monoxide which forms an inactive complex with cytochrome oxidase in the dark. The system is then instantly activated by a flash of light which dissociates the carbon monoxide from the cytochrome oxidase. No mixing is required.

The surprising result is that very high H^+/O ratios are recorded at very times - ratios as great as 50 for the oxidation of succinate. At a time when others would have begun their measurements the H^+/O has fallen to the 4-8 range. These new observations suggest that the H^+ gradient is not mechanistically coupled to electron transport but that the quantity of H^+ translocated depends on the energy available, the existing pH gradient and existing potential gradient across the membrane. Unless initial rates of H^+ translocation and O_2 consumption are measured at every early times, the pH gradient that builds up probably creates a back pressure that greatly reduces the rate of H^+ translocation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00401-18 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Potentiometric studies of respiratory components of E. coli and mitochondria

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard W. Hendler	Section Head	LCB, NHLBI
	K.V.S Reddy	Visiting Fellow	LCB, NHLBI

COOPERATING UNITS *(if any)*

Richard I. Shrager	Mathematician	LAS, DCRT
Barry Bunow	Biomathematician	LAS, DCRT
John S. Rieske	Professor	Ohio State U
Winslow S. Caughey	Chairman, Dept. Biochem.	Colorado SU

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Membrane Enzymology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☑ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have found that cytochrome c1 in beef heart mitochondria behaves as two distinct thermodynamic species. One has an Em 200 mV and a spectral peak at 555 mm and the other has an Em 250 mV and a peak at 553 nm. Both exhibit n values of 2. Current views are that cytochrome c1 is a single thermodynamic entity with Em 230 mV, a peak at 553 nm, and an n value of 1. Our ability to resolve the two species is based on our unique procedures which collect and analyze much more data than is normally used. Our findings are confirmed by two independent analyses. Using a limited amount of data and the older analytical procedure yields the traditional findings for a single species.

A theoretical analysis of Q-cycle mechanisms in respiration, in terms of thermodynamics and kinetics, has been completed.

Initial studies with mitochondrial and purified cytochrome aa3 indicate the presence of three thermodynamic species with Em values of 190 mV, 250 mV and 340 mV and n values of 2, 2, and 1, respectively.

Two new experimental systems were developed. One performs computer-controlled Coulometry while collecting complete optical spectra and the other performs potentiometric titrations using working voltages controlled by a potentiostat.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: To identify and characterize the components of the respiratory chains of E. coli and mitochondria in terms of their redox potentials, numbers of electrons passed, amounts, spectra and possibilities for energy related changes in their properties. The main purpose for acquiring this information is to try to understand the structure of the electron transport chain and the mechanism for transduction of energy, liberated by oxidation, into metabolically useful forms.

Methods Employed and Major Findings: The new techniques for performing and analyzing potentiometric titrations of respiratory chain components, developed in this laboratory, have been applied to a study of cytochrome c_1 of beef heart mitochondria. Our main finding is that cytochrome c_1, which appears to be a single species with an E_m of 230 mV and capable of transferring only one electron at a time when analyzed by the older methods which use only two spectral points in the analysis, is really two thermodynamic species with E_m values of 200 mV and 255 mV, each of which passes two electrons at a time. The two species are also distinguished spectrally, one with a peak at 553 nm and the other at 555 nm. The ability to resolve the seemingly single species into two components by each of two newer methods of analysis is based on the fact that each of the two newer methods is capable of analyzing more data which is unique to the cytochrome of interest. The additional data is available because, instead of recording only a ΔA between a peak and reference wavelength for cytochrome c_1, our procedures record and utilize either an entire peak (13 points) or an entire spectrum (203 points). We have examined the resolving power of the 2-point ΔA method with respect to the problem posed by the particular mixture of c_1 cytochromes that is present and find that with the normal amount of noise that is present in the measurements, the 2-point method simply lacks the resolution to distinguish the components.

A joint theoretical paper on the thermodynamics of the respiratory coenzyme Q cycle was expanded to consider kinetic aspects. This effort, undertaken in collaboration with Drs. Bunow and Rieske, was completed and presented at the Gordon Conference on Bioenergetics and submitted and accepted for publication. Briefly stated, it points to the fact that the ubisemiquinone formed as a result of the one electron reduction of the Rieske iron sulfur center is not necessarily a spontaneous reductant of cytochrome b_T as formulated in the Q-cycle. It also points to kinetic problems made apparent by calculations, that under the conditions proposed for the formation of the postulated highly reducing ubisemiquinone, only one in a million molecules of quinone would be expected to be in the form of the ubisemiquinone. Additional ad hoc assumptions which are required in order for a Q-cycle to work were specified.

Studies on the spectral and thermodynamic characterization of the 4 metal enzyme, cytochrome aa_3 were started. Preliminary results indicate that instead of two thermodynamic species, each passing a single electron (i.e. $n = 1$), there are three thermodynamic species with n values of 2, 2, and 1. This same picture is indicated by studies with intact mitochondria and with the purified enzyme.

The development of two new experimental systems was started this year and both

systems are essentially completed and in the stages of final testing. Because of the indications that cytochrome aa_3 may have three redox transitions with n values of 2, 2, and 1, the possibility exists that the enzyme requires five electrons instead of four for its total reduction. The current view is that only 4 electrons are involved because only 4 metals are present and because only 4 electrons are needed in the reduction of O_2 to 2 H_2O, which is the reaction catalyzed by this enzyme. On the other hand, several laboratories have speculated that in order to accomplish the overall reduction of oxygen, a fifth redox center in the molecule is required. Both a ferryl iron (Fe^{IV}) and a free radical have been suggested as possibilities for this fifth site. In order to answer this question we have had a potentiostatic circuit built by BEIB. This device can hold a known voltage on an electrode and also measure the precise amount of electricity in terms of Coulombs that is used to oxidize or reduce a component in solution. We have written new computer programs which operate the device to administer measured amounts of electricity, to obtain redox equilibrium between charge injections, and to take and record complete spectra after each charge injection. Richard Shrager is developing new mathematical analytical techniques to utilize the new data in order to determine the spectral changes that occur after the injection of the equivalent of 2, 2, and 1 electrons per molecule of enzyme.

The success of the approach depends on having ultra pure enzyme so that the amount of electricity per molecule can be accurately determined. Winslow Caughey at Colorado State University is widely recognized as an expert on the chemistry of cytochrome aa_3 and the producer of one of the purest and best characterized forms of the enzyme. He is providing us with all of the enzyme needed for this study. At the moment we are testing the equipment with known amounts of pure cytochrome c and several different kinds of mediator-titrants.

The second new system also uses the potentiostat. All of our earlier electrochemical studies used a constant current device for accomplishing oxidations and reductions. Our studies with highly purified cytochrome aa_3 have presented new problems. In mitochondria, the cytochromes are isolated in the membranes of the particulate mitochondria. With a purified enzyme in solution, direct contact is made with the electrode. The possiblities for damage to the purified cytochrome may be more pronounced with cytochrome oxidase than with other cytochromes because it requires higher voltages in its oxidation than do the other cytochromes. In order to fix the voltage of the working electrode at values sufficient for oxidation of the enzyme but low enough not to cause any possible damage, a potentiostatic device is required. We have written new computer programs that allow us now to replace the constant current device with the potentiostat.

Biomedical Significance: Cell viability and health depend on the maintenance of adequate electric membrane potentials and energy supplies. Ischemia and anoxia lead to irreversible cell and tissue damage. A basic understanding of the molecular processes by which cells maintain adequate energy and electric balances may lead to means for preventing excessive damage under

conditions of borderline levels of oxygen supply.

Proposed Course of Research: Completion of the spectral and thermodynamic characterization of cytochromes aa_3.

Publications:

1. Reddy, K.V. Subba and Hendler, R.W.: Complete analysis of the cytochrome components of beef heart mitochondria in terms of spectra and redox properties. The b-type cytochromes. J. Biol. Chem. 258:8568-8581, 1983.

2. Hendler, R.W., Bunow, B. and Rieske, J.S.: Thermodynamic and kinetic considerations of Q-cycle mechanisms and the oxidant-induced reduction of cytochromes b. J. Bioenerg. and Biomembr. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00409-14 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interaction of Actin and Myosin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Evan Eisenberg	Head, Section on Cellular Physiology	LCB, NHLBI
Others:	John A. Evans	Staff Fellow	LCB, NHLBI
	Joseph M. Chalovich	Staff Fellow	LCB, NHLBI
	Lois E. Greene	Research Chemist	LCB, NHLBI
	José Biosca	Visiting Fellow	LCB, NHLBI

COOPERATING UNITS *(if any)*
Bernhard Brenner, Visiting Fellow, LPB, NIADDK, NIH, Bethesda, MD 20205
P. Boon Chock, Section Head, LB, NHLBI, NIH, Bethesda, MD 20205

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	4.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have proposed a cross-bridge model of muscle contraction in which during each cycle of ATP hydrolysis, the myosin cross-bridge alternates between a state which binds strongly to actin and a state which binds weakly to actin. Relaxation occurs when the transition between the two states is blocked. In the present study, we have tested several aspects of this model using myosin subfragment-one (S-1) cross-linked to actin; S-1 cross-linked to actin behaves kinetically as if it were at infinite actin concentration. Most important, our results show that, over a wide range of temperature and ionic strength, the rate-limiting step in the acto-S-1 ATPase cycle is equal to the rate at which a muscle fiber redevelops force after a quick stretch. This provides evidence that, as we proposed previously, the rate-limiting step in the acto-S-1 ATPase cycle *in vitro* controls the curvature of the force-velocity curve determined *in vivo*. We also studied O-18 exchange during ATP hydrolysis by the cross-linked S-1 and our results suggest that, if our kinetic model is valid, rotation of the phosphate group at the active site of myosin may be inhibited by the presence of actin.

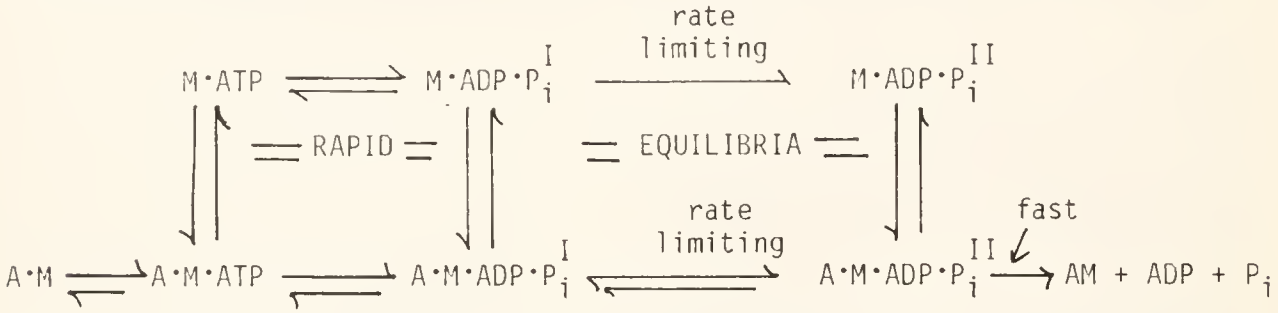
PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives: The accepted mechanism for the contraction of muscle in vivo involves the interaction of actin and myosin filaments. However, the exact nature of the energy transduction mechanism by which the energy stored in the ATP molecule (relative to ADP and P_i) is converted to useful work is not well understood. By studying the biochemical interaction of actin and myosin in vitro we hope to gain insight into the in vivo energy transduction mechanism.

We have proposed the following model for the actin-activated myosin subfragment-1 (S-1) ATPase activity, based on pre-steady-state and steady-state studies of the binding of S-1 to actin in the presence of ATP, and the fact that no significant inhibition of the actin-activated ATPase by actin has ever been observed:



where M = myosin and A = actin. This model suggests that, after ATP binds to actomyosin, the binding of myosin to actin becomes weakened by more than four orders of magnitude. While myosin is in the weak binding state, ATP hydrolysis occurs at the active site and then a rate-limiting conformational change occurs. We have proposed that both the ATP hydrolysis step and the rate-limiting step occur at about the same rate whether the myosin is bound to or dissociated from actin. Following the rate-limiting step, we have proposed that P_i release is rapid and is associated with a conversion of the myosin molecule back to the strong binding state.

One aspect of this model, about which there is still disagreement, is the nature of the rate-limiting step. It has been proposed that the rate-limiting step is the transition from A·M·ATP to A·M·ADP·P_i rather than the subsequent conformational change from A·M·ADP·P_i^I to A·M·ADP·P_i^{II}. Another question of importance is to determine whether the behavior predicted by this model occurs in single rabbit muscle fibers.

Methods and Findings: One prediction of our cross-bridge model is that the rate-limiting step in the ATPase cycle in vitro (V_{max}) should be related to the velocity of muscle contraction in vivo. This is because in our cross-bridge model this rate-limiting step determines the maximum rate at which a cross-bridge can enter the strong-binding state which is also the major force-producing state in the cross-bridge cycle. It is nearly impossible to determine V_{max} under physiological conditions because in the presence of ATP, S-1 binds to actin very weakly. However, by cross-linking S-1 to actin it is possible to determine V_{max} under a wide variety of conditions. There-

fore, we have now been able to determine V_{max} over a range of temperature from 5° to 35° and over a range of ionic strength from 0.012 M to 0.17 M. Our results show that temperature has a marked effect on V_{max}; the Q_{10} is close to 5. However, V_{max} is relatively insensitive to change in ionic strength; there is only about a factor of two increase in V_{max} from μ = 0.012 M to 0.17 M.

Having determined the rate-limiting step in the ATPase cycle over this wide range of conditions, we were able to compare it with the rate of force development in a muscle fiber. This measurement was made by Dr. Bernhard Brenner using his technique of rapidly releasing and then restretching an isometric muscle fiber to detach all of the force-producing cross-bridges. The rate of redevelopment of force can then be determined. We found that from 5° to 35° and from μ = 0.05 M to μ = 0.17 M the rate of redevelopment of force was within a factor of two of V_{max} measured *in vitro*. These data strongly suggest that the rate-limiting step in the ATPase cycle *in vitro* controls the rate of force development *in vivo* and in this way controls the curvature of the force velocity curve.

We have also investigated the rate of O^{18} exchange with cross-linked S-1. Exchange of O^{18} with O^{16} on phosphate produced during ATP hydrolysis is thought to occur during reversals of the ATP hydrolysis step in the ATPase cycle i.e. during the occurrence of the reverse transition from A·M·ADP·P_i to A·M·ATP. If phosphate rotation at the active site is faster than this reverse transition, an O^{16} will exchange for O^{18} during each reversal. In preliminary experiments we have observed almost no O^{18} exchange with S-1 cross-linked to actin. Our model suggests that the reverse transition from A·M·ADP·P_i to A·M·ATP occurs a number of times in each ATPase cycle because the ATP hydrolysis step *precedes* the rate-limiting step. Therefore our data suggest that actin may inhibit O^{18} exchange by decreasing the rate of rotation of P_i at the active site of S-1 cross-linked to actin.

Significance to Biochemical Research: Understanding the mechanism of the actomyosin ATPase is central to gaining an understanding of muscle contraction as well as many other motile systems. This knowledge, in turn, may have important applications in the study of muscular dystrophy and heart disease.

Proposed Course of Research: During the next year, we plan to continue our investigation of O^{18} exchange both with S-1 cross-linked to actin and with S-1 at high actin concentrations. By working under a variety of conditions we hope to confirm our hypothesis that O^{18} exchange is blocked by actin because it affects rotation of P_i at the active site of myosin. We also plan to begin a study of the binding of ATP analogs such as ADP, AMPPMP and PP_i to cross-linked S-1, myosin filaments, and myofibrils and compare this binding with the effect of these analogs on relaxation of force in single skinned rabbit muscle fibers.

Publications:

1. Chalovich, J.M., Chantler, P.D., Szent-Gyorgyi, A.G. and Eisenberg E.: Regulation of Molluscan Actomyosin ATPase Activity. J. Biol. Chem. 259: 2617, (1984).

2. Stein, L.A., Chock, P.B. and Eisenberg, E.: The Rate-Limiting Step in the Actomyosin Adenosinetriphosphatase Cycle. Biochemistry 23: 1555, (1984).

3. Chalovich, J.M., Stein, L.A., Greene, L.E. and Eisenberg, E.: Interaction of Isozymes of Myosin Subfragment-1 with Actin. Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00413-08 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Effect of Tropomyosin on Acto Myosin Subfragment-1 ATPase Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	David L. Williams, Jr.	Staff Fellow	LCB, NHLBI
	Evan Eisenberg	Section Head Cellular Physiology	LCB, NHLBI
Other:	Lois E. Greene	Research Chemist	LCB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.9	1.6	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulatory protein tropomyosin has been shown to confer cooperativity on the binding of myosin subfragment-1 (S-1) to actin and on the actin S-1 ATPase activity. The effect of tropomyosin on the actin-activated S-1 ATPase activity and the effect of tropomyosin on the steady-state binding of S-1 to actin in the presence of ATP were measured at both low and high ratios of S-1 to actin. At low ratios of S-1 to actin, tropomyosin was found to decrease the maximum ATPase rate and to increase the actin concentration required to reach half-maximal activity by about 3-fold in each case. In addition, tropomyosin was found to have little effect on the steady-state binding of S-1 to actin in the presence of ATP. S-1 which had been extensively modified by N-ethyl malemide (NEM) exhibits a very low ATPase activity and is not dissociated from actin by ATP. We used NEM-S-1 to achieve the high ratios of bound S-1 to actin necessary to shift the tropomyosin-actin filament to the potentiated state. We then measured the ATPase of this state using a small amount of unmodified S-1. Under these conditions, tropomyosin had very little effect on the maximum ATPase rate, but the actin concentration required to reach half maximal activity was reduced about 5-fold. Little effect was observed on the steady-state binding in the presence of ATP. Thus, as tropomyosin-actin shifts from inhibited to potentiated the maximum ATPase rate is increased about 4-fold and the amount of actin required to reach half maximal activity is increased about 12-fold, while the steady-state binding of S-1 to actin in the presence of ATP remains constant. Since our previous work shows that tropomyosin may act like troponin-tropomyosin in the presence of calcium, these results suggest even after calcium activation of muscle, the number of force-producing bridges present may greatly modulate the force and velocity exhibited by a muscle fiber.

200

Project Description:

Objectives: The overall objective of this project is to understand how the regulatory protein tropomyosin affects the acto S-1 ATPase in terms of the kinetic parameters V_{max}, K_{ATPase} and the constant describing the steady-state binding of S-1 to actin in the presence of ATP ($K_{binding}$). Skeletal muscle tropomyosin confers cooperativity on both acto-S-1 binding (Williams, D.L., Jr. and Greene, L.E. (1983) Biochemistry 22:2770-2774) and the acto-S-1 ATPase activity (Bremel, R.D. and Weber, A. (1972) Nature (London), New Biol. 238, 97-101; Bremel, R.D., Murray, J.M. and Weber, A. (1972) Cold Spring Harbor Symp. Quant. Biol. 37:267-275). Recent work indicates the effect of tropomyosin on actin-S-1 binding may be related to its effect on the acto-S-1 ATPase activity (Williams, Greene and Eisenberg, 1984). Tropomyosin inhibits the acto·S-1 ATPase at low S-1 to actin ratios. As the S-1 concentration is raised, the effect of tropomyosin goes from inhibitory to activating, due to bound S-1 shifting the tropomyosin-actin to a different state. The effects of tropomyosin on the acto·S-1 ATPase activity were studied at low ratios of S-1 to actin (where tropomyosin has an inhibitory effect) and at high ratios of S-1 to actin (where tropomyosin has a potentiating effect). The effect of tropomyosin on the steady-state binding of S-1 to actin in the presence of ATP was also investigated at both ratios of S-1 to actin.

Methods Employed and Major Findings: The acto·S-1 ATPase activity was measured in the presence and absence of tropomyosin at several actin concentrations at 12 mM ionic strength, 15°C. The amount of radioactivity released from [γ ^{32}P]ATP per second was used to determine the ATPase rate. The S-1 to actin ratio was kept very low to ensure that ATP-free (rigor) myosin heads were not perturbing the tropomyosin-actin complex. Alternatively, high ratios of N-ethyl maleimide-treated S-1 to actin were used to ensure that ATP-resistant (rigor-like) myosin heads with very low ATPase activity maximally potentiated the tropomyosin-actin complex. The ability of this potentiated complex to activate the S-1 ATPase activity was then measured by adding a small amount of unmodified S-1. The steady state binding of S-1 to tropomyosin-actin in the presence of ATP was measured by mixing the S-1 with varying concentrations of the tropomyosin-actin complexes. After centrifuging the tropomyosin acto·S-1 complex, the concentration of S-1 which remained in the supernatant was measured by determining its ATPase activity. From the amount of S-1 in the supernatant, we could determine the extent of S-1 binding.

Our results show that, at low ratios of S-1 to actin, tropomyosin decreases V_{max} and weakens K_{ATPase} about 3-fold in comparison to unregulated actin. Then, when the tropomyosin-actin activated S-1 ATPase activity goes from inhibited to potentiated, the K_{ATPase} is strengthened about 12-fold and V_{max} increases about 4-fold. Tropomyosin was observed to have little effect on the steady-state binding of S-1 to actin in the presence of ATP in comparison to unregulated actin. Since our previous work shows that tropomyosin may act like troponin-tropomyosin +Ca^{2+}, these results suggest even after Ca^{2+} activation of muscle, the number of force-producing bridges present may greatly modulate the force and velocity exhibited by a muscle fiber.

Significance to Biomedical Research: The interaction of actin with myosin in the presence and absence of tropomyosin provides information on the basic mechanism of muscle contraction and its regulation. An understanding of the regulation of contraction is critical in the study of diseases of skeletal, cardiac, and vascular muscle.

Proposed Course of Research: The next step in this project is to investigate the effect of troponin-tropomyosin on the acto·S-1 ATPase kinetic parameters both in the presence and absence of Ca^{2+} and at both low and high ratios of S-1 to actin. First, the V_{max} and K_{ATPase} of the troponin-tropomyosin-acto-S-1 ATPase will be determined at low ratios of S-1 to actin, in the presence and absence of Ca^{2+}. Next, these same parameters will be determined for the troponin-tropomyosin system with and without Ca^{2+} at high NEM-S-1 to actin ratios. Steady-state binding in the presence of ATP will be investigated using NEM-S-1 to potentiate the tropomyosin-actin complex. NEM-S-1 will also be used to study the potentiation of cross-linked S-1-actin ATPase activity in the presence of tropomyosin and troponin-tropomyosin.

Publications:

1. Williams, D.L., Jr., Greene, L.E., and Eisenberg, E. Comparison of the effects of smooth and skeletal muscle tropomyosin on the interactions of actin and myosin subfragment-one. (1984) Biochemistry, (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00418-04 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Electrochemical Potentials of Protons in Energy-Tranducing Membranes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Richard W. Hendler	Section Head	LCB, NHLBI
	Oruganti H. Setty	Visiting Associate	LCB, NHLBI

COOPERATING UNITS (if any)	Richard I. Shrager	Mathematician	LAS, DCRT
	Baltazar Raynafare	Research Associate	JOHNS HOPKINS UN
	Albert L. Lehninger	Professor	SCH OF MEDICINE
	Jeffrey P. Froehlich	Medical Officer	NIA, NIH

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two new systems have been devised that allow the measurements of zero-time values of H+/O ratios during respiratory pulses. This zero-time value, thus far undetermined, is important in understanding the conversion of respiratory to metabolic energy. One system uses mitochondria exposed to substrate and oxygen, but prevented from respiration by a carbon monoxide atmosphere in the dark. A quick exposure to light releases the inhibition and causes a burst of respiration. A microcomputer collects data at 0.01 sec intervals. Computer analysis is used for noise reduction, corrections for relaxation times of the electrodes and determination of the time course of the ratio. Results obtained with the system do not support Mitchell's views of energy conversion at sites II and III of the respiratory chain. A previously undescribed "burst phenomenon" appears to operate in the form of an immediate discharge of a large number of protons per atom of oxygen consumed at zero time.

A second system has been developed. This system uses pH and oxygen electrodes placed in a flow cell at fixed distances from a rapid mixing device. The mitochondria are instantly mixed with oxygen in the device and the electrodes continuously sample the suspension at different times of respiration on a scale of milliseconds to 1 sec depending on flow velocities and distance of the electrodes from the point of mixing.

203

Project Description:

Objectives: To study the interrelation of important parameters of energy transduction during pulses of succinoxidase activity in respiratory vesicles and to determine H^+/O ratios under a variety of metabolic conditions.

Methods Employed and Major Findings: All activities in this project have been focused on devising experimental approaches to determine the ratio of H^+ translocation to oxygen uptake as near as possible to zero time of a respiratory pulse. This is important in order to define the mechanism of conversion of respiratory energy into ATP. Peter Mitchell's mechanism locks the translocation of H^+ to the transport of electrons in the respiratory chain in such a way that the H^+/O ratio for electrons introduced from succinate must be 4.0. Lehninger and associates favor a mechanism which sets the ratio at 8.0, whereas other laboratories predict 6.0. All agree that 4 protons are transported at energy transduction site II (cyt b to cyt c_1). The disagreement centers on site III (cytochrome oxidase) where Mitchell says no protons are translocated, Lehninger says 4 and others say 2. The experimental problems which have prevented a clear resolution of this question over the course of many years are: 1) As soon as the first H^+ are translocated, the developing membrane potential hinders other H^+ from following, thus lowering the measured H^+/O ratio. 2) Existing techniques which initiate respiration with an injection of oxygenated solution have a built-in dead time of 0.6 to 1 second due to mixing. 3) The initial changes in signals from the pH and oxygen electrodes are in microvolts and therefore difficult to distinguish from electrical noise. 4) Electrodes have a characteristic delay in response (relaxation time) which is critical in these early measurements and no one has made proper corrections for this phenomenon. During this past year we have successfully dealt with all of these problems and have devised two different experimental systems for obtaining zero time and early measurements of the H^+/O ratio in rat liver mitochondria. The development of these systems followed an initial approach designed to decrease the speed of mixing injected oxygenated buffer. It soon became apparent that even under the best conditions a dead time of at least several tenths of seconds must be present. New approaches were clearly required. One of the two systems we developed is based on the idea that a well mixed mitochondrial suspension provided with substrate and oxygen will not respire in the presence of saturating carbon monoxide when kept in the dark. Exposure of this system to intense light, photolyzes the carbon monoxide-cytochrome oxidase complex, initiating a pulse of respiration. In order to use this system it was necessary to drastically reduce noise levels seen by the electrodes. This process involved the identification of different noise sources. A combination of measures led to a very marked lowering of the background noise. 1) A combination pH electrode has a level of noise due to the movement of currents of stirred liquid across the junction of the reference electrode cell, which causes fluctuations in the junction potential. This source of noise was removed by using separate pH and reference electrodes and by placing the latter in a separate unstirred vessel. Electrical connection between the stirred and unstirred vessels was established with an Agar/buffer bridge, cast in a polyethylene tube. 2) The experimental

set-up was shielded from ambient electrical noise by being placed in a grounded metal box (Faraday cage). 3) 60-cycle electrical noise was markedly reduced by putting salt in the water bath which circulated in the jacket around the reaction chamber. 4) A series of low-frequency-pass electronic filters was used. 5) Data was collected at a density of 100 points per electrode per second so that final smoothing was accomplished during computer fitting of the data.

The relaxation time for each electrode was experimentally measured at the time of the experiment. This involved the design and construction of a special flow cell. To determine relaxation time, either pH or $[O_2]$ must be abruptly changed. Injection of H^+ or O_2 into a stirred solution can not be used because of mixing times. In the flow cell each electrode is exposed to a 1 mm central channel which is first filled with a solution at one pH or $[O_2]$. The solution is abruptly displaced by new solution at a different pH or $[O_2]$, fed by an attached syringe. The response of the electrode is used to compute the relaxation constant, τ. The true rate of change (dZ/dt) of concentration of H^+ or O_2 is related to the observed rate (dY/dt) by the expression:

$$\frac{dZ}{dt} = \frac{dY}{dt} + \tau \frac{d^2Y}{dt^2}$$

The actual experiment for determining initial H^+/O ratios is done as follows. Data are collected from each electrode every 0.01 sec from just before to several seconds after opening a photographic shutter to initiate the respiratory pulse. The raw data are computer-fit to suitable exponential functions. This procedure both smooths the data by averaging and also represents all of the data by two known equations (for H^+ and O_2). The first and second derivatives of each equation with respect to time are taken. Knowing dY/dt, d^2Y/dt^2, and τ enables us to express true dH^+/dt and dO/dt at any time by using the equation shown above. The ratio of dH^+/dt to dO/dt gives the true H^+/O value at any time. Experiments with succinate as electron donor have been conducted and are currently under analysis. The findings, so far, are most unexpected. Initial H^+/O ratios appear to be very high (some >50) and there is a rapid decline so that after several tenths of a second, values below 8 are seen. There seems to be little doubt that Mitchell's view of a process which accounts for only 4 protons per atom of oxygen is incorrect. The view of a single process yielding a fixed ratio of 8 is also inadequate. Although more work is required before a final position can be taken, there are two new concepts suggested by the apparent high initial ratios. One is that the release of energy by the respiratory chain is a separate process from the pumping mechanism which uses that energy to move protons. In such case the number of protons translocated would be determined by the existing pH gradient and $\Delta\Psi$ across the membrane and the amount of energy available for pumping. At the start of respiration where little or no $\Delta \bar{\mu}_{H^+}$ exists, a very large number of H^+ could be moved at little energy cost. Another possibility is that a basic process which translocates a fixed number of protons per atoms of oxygen (8) is augmented by an initial

burst whereby a reservoir of membrane-sequestered or Bohr-type associated protons are liberated. The reservoir would be replenished during low energy or non-respiratory conditions.

The second system developed to measure directly the zero or early time H^+/O ratios employs a continuous flow cell and rapid mix technology. One syringe contains anaerobic mitochondria plus succinate and another contains buffer with limited oxygen. A special pump forces the contents of the two syringes through a Berger ball mixing device for instantaneous mixing. The oxygen and pH electrodes held in a flow cell designed for these experiments are spaced at precise distances from the zero-time point of mixing to be continuously exposed to mitochondria that have been respiring for fixed times on a scale of milliseconds to 1 sec. No corrections for response times of the electrodes are required because the flow lasts long enough to allow the electrodes to attain stable readings. As was the case in the development of the carbon monoxide system described above, various special measures had to be taken to reduce background noise sources to minimal values. This system has passed most of the test situations we have devised and will soon be used for actual experimental measurements with mitochondria.

Proposed Course of Research: To further develop the two new methods described in this report and apply them to the determination of H^+/O ratios from zero time through several seconds of a respiratory pulse with particular emphasis on the characteristic ratios at sites II and III of the mammalian respiratory chain.

Publications:

1. Setty, O.H., Hendler, R.W., and Shrager, R.I.: Simultaneous measurements of PMF, ΔpH, $\Delta\Psi$, and H^+/O ratios in intact E. coli. Biophys. J., 43:371-381, 1983.

2. Hendler, R.W., Setty, O.H., Shrager, R.I., Songco, D.C., and Friauf, W.S.: Instrumentation and procedures for real time measurements of proton motive force, membrane potential, ΔpH, proton extrusion, and oxygen uptake in respiring cells and vesicles. Rev. Sci. Instrum. 54:1749-1755 (1983).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00419-04 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure-Function Relationships in Eukaryotic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Blair Bowers	Research Biologist	LCB, NHLBI
Others:	Enrico Cabib	Senior Research Chemist	LBM, NIADDKD
	Martin L. Slater	Health Sciences Admin.	DRG, NIH
	Kyung Nam	Biologist	LCB, NHLBI

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two studies related to yeast wall structure examined (1) the role of mannoproteins in cell wall premeability and (2) the function of cell division cycle (cdc) gene 3 in septum formation. These studies were performed in collaboration with the laboratory of E. Cabib, NIADDKD. The first study showed that mannoproteins which coat the outer surface of the yeast cell wall function in part to control cell wall porosity and hence some to protect the plasma membrane from external lytic agents. In the second study we examined yeast with a temperature sensitive mutation of cdc gene 3, which results in failure of cytokinesis. At the non-permissive temperature the mutant cells form septa which are similar in structure and chemical composition to normal septa. However, the septa fail to form at the junction of the bud and mother cell, but rather cut off peripheral bits of cytoplasm at their aberrant locations. Results with double mutants showed that septum formation requires prior events of DNA synthesis and nuclear division, and that the cdc 3 gene product is required for correct location but not the formation of the septum.

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Project Description:

Objectives: These studies, conducted on two different eukaryotic systems, are in each case directed toward understanding the control mechanisms of a cellular event that has a morphologically identifiable end point. We are examining factors that limit or accelerate endo- and exocytosis in Acanthamoeba. These studies have particular relevance to two areas of current interest in the Laboratory, namely membrane recycling, which appears to be tied to endocytic events in Acanthamoeba, and the association of actin with membranes, since the endocytic event appears to require intermittent "recruitment" of actin to a particular membrane site. In other studies (in collaboration with E. Cabib, NIADDKD) the long range objectives are to elucidate the way in which yeast chitin synthetase, an enzyme found generally on the plasma membrane, can be selectively activated at only one site on the membrane to cause a localized deposition of chitin.

Methods Employed: Transmission electron microscopy is being used for morphological studies of fixed and embedded cells and for examination of purified proteins by negative staining. Phase and fluorescence light microscopy are used to monitor living cells. Standard biochemical procedures are used for enzyme activity and protein measurements. Cytochemical staining of thin sections was performed using colloidal gold stabilized with appropriate ligands.

Major Findings: 1) The yeast cell wall is composed primarily of polymers of glucose and mannose. The glucans appear to provide the major structural scaffolding of the cell wall. The mannans, linked by small amounts of peptides, cover the outer surface of the wall. Mannan function is not well understood. A protease was purified from a yeast cell wall lytic mixture (called zymolyase) that is secreted by a bacterium. The protease was demonstrated to be specific for mannoproteins. Digestion of the intact yeast with the protease removed the outer wall layer, concomitantly solubilizing virtually all the wall mannan. Removal of the outer wall layer did not affect cell shape but increased the permeability of the yeast cell wall. It was demonstrated that the cell wall glucan could be hydrolyzed by glucanase and that horseradish peroxidase (Mr 40,000) could penetrate to the plasma membrane after mannan removal. In intact cell walls molecules larger than about 700 molecular weight do not penetrate the wall. This work demonstrates a role for the externally disposed mannoproteins in controlling cell wall porosity.

2) Last year in a study of cell division cycle (cdc) mutants in yeast, we described the effects of several cdc mutations on chitin synthesis. One of those mutants, cdc 3, fails to undergo cytokinesis although budding, and nuclear division take place. Cytokinesis normally occurs through the formation of a thin chitinous septum separating the mother and daughter cells. We found that cdc 3 mutants formed structures that resemble normal septa except they were spatially misplaced. They occurred around the periphery of the cell wall, cutting off small bits of cytoplasm, but did not form at the appropriate site to separate the bud from the mother cell. Chitin-specific cytochemical stains showed that the aberrant septa contained chitin. The cdc 3 mutation was combined in a series of double mutants with temperature-sensitive mutations affecting start function (cdc 28), bud emergence (cdc 24), initiation (cdc 4) or continuation (cdc 24) of DNA synthesis or nuclear division (cdc 13). The cdc 4 mutation, for example, allows cell

growth and budding, with apparently normal wall formation but not initiation of DNA synthesis. It is independent of the cdc 3 mutation which also allows growth and budding but not cytokinesis. None of the double mutants formed the aberrant septa, indicating that the formation of the septa requires specific functions of the paired mutations including DNA synthesis and nuclear division. The results show that the particular geometry of the neck region is not required for the formation of the septal plate. It is concluded that formation of the aberrant septa is controlled by the same sequences of cell cycle events as normal septa, but that the spatial controls that place the septum at the appropriate junction between mother and daughter cell have been lost in the cdc 3 mutant.

3. Several electron microscopic studies have been carried out for members of the Laboratory of Cell Biology. (a) With Jacek Kuznicki, we have examined, by negative stain and rotary shadowing, the molecular size and configuration of myosin II. Untreated myosin was compared with myosin treated with chymotrypsin to cleave the phosphorylation sites of the tail region. A number of variables, including buffer type, pH, and Mg concentration were examined. The electron micrographs showed that chymotrypsin cleaved myosin did not form bipolar filaments under conditions which allowed filament formation in untreated myosin, but did form monopolar dimers or larger aggregates. Measurements of tail lengths of chymotrypsin-treated and untreated monomers showed that the tails of chymotrypsin cleaved monomers were about 10 nm shorter than those of untreated myosin. (b) We examined actin filament length as a function of time after sonication for M.-F. Carlier. (c) With J.P. Albanesi we attempted to determine if myosin I was associated with the membrane of isolated phagosomes. Transblots of polyacrylamide gels of membrane fractions were made and stained with myosin I antibodies (from J. Hammer) and horseradish peroxidase coupled to goat anti rabbit immunoglobulin. By this criterion phagosomes and the membranes isolated from phagosomes contained myosin I. Isolated plasma membrane fractions did not. Cytochemical localizations with the specific myosin I antibodies and protein A colloidal gold did not show any myosin I antibody binding to phagosomes or to isolated phagosome membranes. The results of the two procedures may indicate that the myosin I found in the isolated membrane fraction is not associated with membranes, but both types of localization need to be repeated before firm conclusions can be drawn.

Significance to Biomedical Research: Phagocytosis is a major mechanism of human defense against infection. This process can be profitably studied in the ameba where experimental conditions are simplified and where phagocytosis is much exaggerated. Certain yeasts are pathogenic in humans. Understanding the control of synthesis of chitin, an important wall component, may aid in developing better therapies for infections.

Publications:

1. Bowers, B. and Olszewski, T.: Acanthamoeba discriminates internally between digestible and indigestible particles. J. Cell. Biol. 97:317-322, 1983.

2. Zlotnik, H., Fernandez, M.P., Bowers, B. and Cabib, E.: In Saccharomyces cerevisiae, mannoproteins form an external layer of cell wall which determines cell porosity. J. Bacteriol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 000501-11 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Actin Polymerization

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Edward D. Korn	Chief, Lab of Cell Biology	LCB, NHLBI
Others:	Stephen L. Brenner	Research Chemist	DCRT
	Martine Coué	Visiting Fellow	DCRT
	Altaf A. Lal	Guest Worker	LCB, NHLBI
	Dominique Pantaloni	Expert/Guest Worker	LCB, NHLBI
	Marie-France Carlier	Guest Worker	LCB, NHLBI
	Joel Vandekerckhove	Professor	State Univ. Ghent, Belgium

COOPERATING UNITS *(if any)*

Division of Computer Research and Technology
Laboratory of Genetics, State University Ghent, Belgium

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.25	5.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

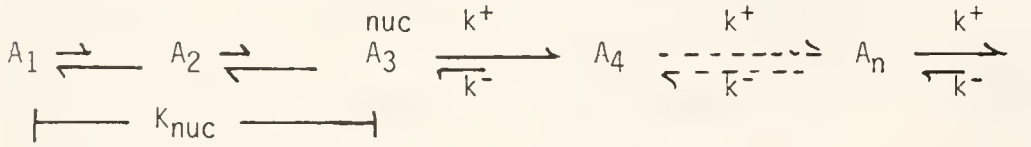
SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism and consequence of ATP hydrolysis accompanying the polymerization of actin has been studied. We have found that ADP·actin polymerizes spontaneously similarly to ATP·actin. By greatly increasing the rate of polymerization of ATP·actin by sonication, we have proved that hydrolysis of ATP occurs as a subsequent step on the F-actin. The critical concentration and association and dissociation rate constants have been determined for actin polymerized in ATP and in ADP. The critical concentration in ADP is greater than in ATP because the association rate constant is lower and the dissociation rate constant is larger. The kinetic constants in ADP are, as expected for an equilibrium polymer, the same above and below the critical concentration. But in ATP, the filaments at steady state have an ATP-cap that stabilizes the polymer. As a result, when the ATP cap is lost dissociation occurs at the higher rate determined by the dissociation rate constant for ADP·actin. When the rate of formation of ADP·actin monomers exceeds the rate of exchange of ATP for ADP on the actin, ADP·actin accumulates and the rate of association is determined by the lower rate constant for ADP·actin. Thus, below its critical concentration or when filament number concentration is greatly increased the rate constants for ATP·actin approach those for ADP·actin. This phenomenon also applies at steady state where a statistical fraction of filaments will lose their ATP caps and rapidly depolymerize while ATP-capped filaments elongate in equivalent amount. Thus, the energy of hydrolysis of ATP stabilizes the actin filament, which contains mostly ADP·actin subunits and an ATP·actin cap, and allows regulation of the polymerization state through changes in the rates of individual steps in the polymerization process. Non-covalent modification of actin by virtue of associated ATP or ADP is, therefore, exactly analogous to covalent modification of proteins through phosphorylation and dephosphorylation.

Project Description:

Objectives: Actin is a major component of all eukaryotic cells where it has an essential role in maintaining cell structure and, with myosin, in many important motile events. Actin is a monomeric protein of Mr 42,000 that polymerizes to long helical filaments which are the function state of actin. In non-muscle cells, especially, the state of polymerization in under active regulation. We are studying the regulation of the polymerization of actin by other proteins, isolated from cells, that interact with either actin monomers or actin polymers or both. In addition, one mole of actin-bound ATP is hydrolyzed to actin-bound ADP for every mole of actin that is converted from monomer to polymer. The regulatory role of this ATP hydrolysis has been a major part of our effort this year.

Methods Employed and Major Findings: Previously, we followed the polymerization of actin by the increase in fluorescence of a pyrenyl group attached to cys-374, and, by computer fitting of the experimental data to theoretical models, established the general nature of the polymerization process. Polymerization could be described as a nucleation-elongation process in which monomers are in an unfavorable pre-steady state equilibrium with trimers (the nucleus for polymerization) and trimers rapidly elongate to polymers by addition of monomers to both ends of the growing filaments.



This year we used actin trimers covalently crosslinked by phenylbismaleimide to nucleate polymerization, thus avoiding the slow nucleation phase. We found that the rate of polymerization was directly proportional to the trimer concentration and to the monomer concentration. This allowed us to convert the observed rate of polymerization to an association rate constant, k^+ because the trimer and monomer concentrations were both known (in contrast to spontaneous polymerization in which the concentration of growing filaments is not known). The critical concentration, i.e. the concentration of actin monomer when polymerization is at steady state, was determined independently from the fluorescence at steady state as a function of total actin concentration. Because the critical concentration, Cc, equals k^-/k^+, it was then possible to calculate the dissociation rate constants, k^-. When polymerization was initiated in 1.0 mM $MgCl_2$, the values were: Cc=0.34 μM, k^+=1.8 $\mu M^{-1}s^{-1}$; k^-=0.6 s^{-1}. In 1 mM $MgCl_2$ + 0.1 M KCl, the values were: Cc, 0.07; k^+, 5.2 $\mu M^{-1}s^{-1}$; k^-, 0.4 s^{-1}.

We then developed a procedure for preparing monomeric actin with bound ADP, instead of bound ATP, and studied its polymerization. From the limited data obtainable for spontaneous polymerization, it was possible to show that the general polymerization kinetics for ADP·actin were very similar to those for ATP·actin: there was a nucleation and elongation phase and the data were consistent with a nucleus size of 3. The Cc for actin in ADP, however, was found to be much higher than for actin in ATP. The Cc in 1 mM $MgCl_2$ was 8 μM

-2//

and in 1 mM $MgCl_2$ + 0.1 M KCl it was 2 μM, 20 to 30 times higher than the values in ATP. By the same procedure of nucleating polymerization with cross-linked trimers, we found that higher critical concentrations were due to lower values for k^+ and higher values for k^-: in 1 mM $MgCl_2$, k^+= 0.8 μM^{-1} and k^-=6.4 s^{-1}; in 1 mM $MgCl_2$ + -.1 mM KCl, k^+=0.9 μM^{-1} s^{-1} and k^-=1.8 s^{-1}. Thus, monomers add more slowly to and dissociate more rapidly from filament ends in ADP than in ATP. This is equivalent to saying that the actin polymer is more stable in ATP than in ADP.

But the consequences of the hydrolysis of ATP during actin polymerization in ATP are even more complicated and more interesting than the above results might suggest. We have found that polymerization of actin in ATP is mechanistically uncoupled from hydrolysis of ATP. Hydrolysis of ATP occurs on the filament after the addition of the actin·ATP subunit is complete. This was shown by measuring the rate of polymerization and the rate of ATP hydrolysis under continuous sonication. Sonication accelerates polymerization by constantly breaking filaments and thus increasing the number of ends to which monomers can add but sonication does not affect the rate of hydrolysis of ATP on the F-actin. Under the conditions used, polymerization (monitored by light scattering or by the increase in fluorescence of NBD-labeled actin) was complete in about 10 seconds while hydrolysis of an equivalent amount of ATP required 200 seconds. The rate constant for the hydrolysis of ATP on the F-actin was about 0.022 s^{-1} in 1 mM $MgCl_2$. As expected for a first order reaction, this rate constant was independent of actin concentration.

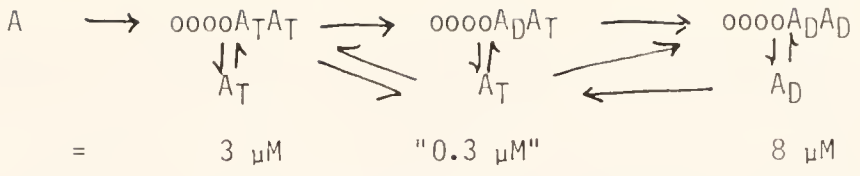
Because ATP hydrolysis occurs on the filament, after addition of the ATP·actin subunit, there will necessarily be an ATP·actin cap at the ends of the filaments while the bulk of the actin subunits in the interior of the filament will contain bound ADP. We found that this ATP cap persists at steady state, when polymerization is complete but monomers and polymers continue to interact. The consequences of this ATP cap can be shown in experiments in which actin filaments at steady state in ATP or in equilibrium in ADP are diluted into different concentrations of monomeric actin. When the monomer concentration is above the Cc, addition of monomers to the polymer ends will occur and, when the monomer concentration is below the Cc, dissociation of monomers will occur. By measuring the rate of change in fluorescence of pyrenyl actin, the k^+ and k^- values for subunit addition and loss can be calculated. When this experiment was done with actin in ADP, the same values for the rate constants were obtained above and below the critical concentration and the values agreed with those determined before from the kinetics of nucleated polymerization.

This is as predicted for an equilibrium polymer. When the same experiment was done in ATP, different values were obtained for the rate constants above and below the critical concentration. Above the critical concentration, the values previously found for the polymerization of actin in ATP were obtained. But when actin in ATP was diluted below its critical concentration, the rate constants approached those found for actin in ADP. At infinite dilution, the same rate constants are found in ATP as in ADP. This occurs because depolymerization of F-actin in ATP exposes internal subunits that have bound ADP and because the exchange of ATP for ADP on the actin is slower than the rate of subunit dissociation.

As a consequence of this behavior of actin in ATP, a very interesting situation develops when actin is at steady state in ATP. Under these conditions, which is the stable situation, the concentrations of polymeric and monomeric actin are maintained constant but association and dissociation reactions at the filament ends continue to occur. The number of associations equals the number of dissociations so that steady state is maintained. But statistical variation among the large population of filaments will result in some filaments losing their ATP cap. When this occurs the filament will dissociate rapidly, according to the k^- for ADP·actin which is much greater than the k^- for ATP·actin. This rapid dissociation of filaments that have lost their ATP caps will be balanced by an equivalently rapid elongation of filaments that are still capped with ATP. Thus, the situation at steady state is much more dynamic than was previously considered and all previous date for monomer-polymer exchange at steady state must be re-interpreted.

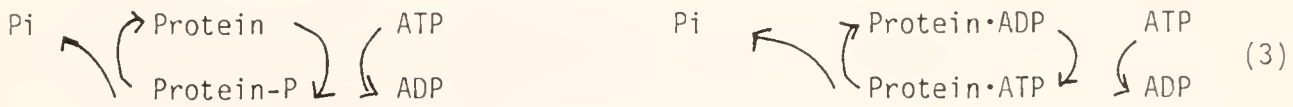
Also, any change in the concentration of filament ends will have a profound effect on the extent of polymerization. We observed that when the filament number concentration at steady state was increased about 20-fold by sonication, the critical concentration increased from that characteristic of ATP·actin to that of ADP·actin i.e., sonication at steady state causes depolymerization in addition to fragmentation. Initially, the system is at steady state with ATP·monomers and ATP-capped polymers. Sonication exposes the internal ADP·actin subunits which then rapidly dissociate with the k^- for ADP·actin. The exchange of ATP for ADP on the filament ends and on the ADP·monomers formed by dissociation in slower than the rate of subunit dissociation from filaments. Therefore, ADP·monomers and ADP·filament ends accumulate instead of ATP·actin subunits and the system approaches the kinetics of actin in ADP, even though excess ATP is present. In addition to its contribution to our understanding of the mechanism of polymerization, the interest in this system is that there are many proteins in cells that interact with actin filaments in such a way as to break them into many smaller filaments. It is thus possible that the action of these filament-severing proteins may be similar to that of sonication. Severing proteins would then not only increase the number of filaments by converting long filaments to short ones but may also decrease the concentration of polymeric actin.

As a result of these and other experiments, we now understand much better the regulatory role of ATP hydrolysis in actin polymerization. By virtue of the ATP cap, the actin polymer is stabilized. Maximal stability requires the energy of hydrolysis of ATP; we have shown that the actin critical concentration is higher (that is, the stability of the polymer is lower) under conditions where ATP hydrolysis does not occur than when it does. A filament that contains only ATP·subunits has a critical concentration intermediate between an all ADP filament and an ATP-capped one. Because ATP hydrolysis is irreversible, the system is at steady state rather than an equilibrium. This allows subtle control of the state of polymerization by regulation of rates. It thus becomes possible to shift rapidly and extensively from the critical concentration and rate constants characteristic of ATP to those of ADP, even though ATP is still present in excess, by changes in monomer concentration or filament number, both of which can be regulated by the interaction of actin with other proteins, or by changes in the rate of any of the steps in the polymerization process.



(2)

The major conclusion, then, is that hydrolysis of ATP regulated the actin system by determining whether ATP or ADP will be bound to the actin (non-covalent modification) in just the same way as hydrolysis of ATP through a kinase/phosphatase cycle regulated other systems (e.g. myosin) by phosphorylation and dephosphorylation of a protein (covalent modification).



(3)

One of the proteins that interacts with monomeric actin and regulates its polymerization is profilin which forms a 1:1 complex with actin monomers. In collaboration with Dr. Joel Vandekerckhove, we have now determined the complete amino acid sequence of this 125-residue protein from Acanthamoeba castellanii. There is considerable homology in the N-terminal 40 amino acids to the somewhat larger mammalian profilin but no homology in the remainder of the sequence. The amino terminal half of the profilin is very hydrophobic while the C-terminal half is especially rich in basic amino acids.

Proposed Course of Research: We intend to study the interaction of several actin-binding proteins both in the presence of ATP and of ADP. Some of the proteins to be studied are tropomyosin and troponin, from muscle, and brevin, which is a plasma protein similar to gelsolin that occurs in cells. Tropomyosin/troponin interact along the entire length of actin filaments while brevin caps one end of the actin filament. We also intend to study the process by which short filaments redistribute into a smaller number of longer filaments in the presence and absence of regulatory proteins. More details of the hydrolysis of ATP by actin at steady state and during polymerization will be explored including determination of many of the rate constants of scheme 3.

Publications:

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Tobacman, L.S., Brenner, S.L., and Korn, E.D.: Effect of Acanthamoeba profilin on the pre-steady state kinetics of actin polymerization and on the concentration of F-actin at steady state. J. Biol. Chem. 258: 8806-8812, 1983.

Vandekerckhove, J., Lal, A.A., and Korn, E.D.: Amino acid sequence of Acanthamoeba actin. J. Mol. Biol. 172: 141-147, 1984

Brenner, S.L., and Korn, E.D.: Evidence that F-actin can hydrolyze ATP independent of monomer-polymer end interactions. J. Biol. Chem. 259: 1441-1446, 1984.

Pantaloni, D., Carlier, M.-F., Coue, M., Lal, A.A., Brenner, S.L., and Korn, E.D.: The critical concentration of actin in the presence of ATP increases with the number concentration of filaments and approaches the critical concentration of actin·ADP. J. Biol. Chem. 259: 6274-4283, 1984.

Lal, A.A., Korn, E.D., and Brenner, S.L.: Rate constants for actin polymerization in ATP determined using cross-linked actin trimers as nuclei. J. Biol. Chem., in press.

Korn, E.D. The regulation of actin and myosin by ATP. In Current Topics in Biochemical Regulation (Eds.) P. Boon Chock and A. Ginsburg, 1984

Carlier, M.-F., Pantaloni, D., and Korn, E.D.: Evidence for an ATP cap at the ends of actin filaments and its regulation of the F-actin steady state. J. Biol. Chem. 1984, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00503-12 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure, Assembly and Function of Microtubules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Martin Flavin	Head, Section on Organelle Biochemistry	LCB, NHLBI
Other Investigators:	Adavi Murthy, Visiting Fellow		LCB, NHLBI
	Charles Patterson, Staff Fellow		LCB, NHLBI
	Shingo Tsuyama, Visiting Fellow		LCB, NHLBI
	Gregory Bramblett, Research Assistant		LCB, NHLBI

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Organelle Biochemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.1	3.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our current project concerns the function of post-translational modifications of microtubule proteins: tyrosinolation of tubulin, and phosphorylation of MAP-2.MAP-2 (MW 270,000) is the principal protein in vertebrate brain (where it is largely confined to dendrites) that coassembles with, and promotes assembly of, tubulin in vitro. MAP-2 as isolated contains about 10 mol of phosphate (A subset). An additional 10 (B subset) can be added by cAMP kinase, a large proportion of which in brain is strongly bound to MAP-2. Two results suggest that A and B phosphates occupy different sites. 1) B phosphates are enriched in the 32-kDa binding domain, whereas A phosphates (those that turn over in vivo) are confined to the 240-kDa projection domain. 2) A phosphates are relatively resistant to all types of protein phosphatase. The practical goal of finding a phosphase with specificity for A sites has been hindered by limited amounts of this labeled substrate. Meantime, using B site labeled MAP-2 as substrate, a brain enzyme has been purified which has relatively high activity with this substrate compared to others (the same is true for brain calcineurin). B site phosphates have been shown to affect microtubule assembly. We wish now to determine the effect of A and B site phosphates on microtubule interaction with neurofilaments and other organelles.

Project Description:

Objectives: Our objectives are to ascertain the biological functions of microtubule modification by tyrosinolation and phosphorylation.

Methods Employed: Biochemical procedures as indicated under Major Findings.

Major Findings:

1. MAP_2 phosphorylation. MAP_2 is the predominant protein (MW 270,000 by SDS-PAGE) in vertebrate brain that coassembles *in vitro* in constant proportion to tubulin, and promotes *in vitro* assembly of pure tubulin. About 1/3 of brain cAMP dependent protein kinase is known to be firmly bound to MAP_2, a remarkably high proportion in view of the current concensus that MAP_2 is largely confined to dendritic processes. A year ago we reported that purified MAP_2 contained 8 to 10 mol phosphate/mol protein (A subset) as determined colorimetrically after wet ashing. Upon incubating assembly-cycle purified microtubule protein with [γ-^{32}P]ATP, 10-12 additional mol of phosphate were added to MAP_2 (other MTP were little labeled) by the endogenous kinase. These (B subset) were in addition to, and not by isotope exchange with, the A phosphates. We purified a protein phosphatase from brain that was found to act preferentially on the B subset phosphates. Using these enzymes, MAP_2 was prepared in low (10 mol/mol) and high (20 mol/mol) phosphorylation states. A number of parameters of MAP_2 - promoted microtubule assembly were shown to be affected by its phosphorylation state. During the past year we have focussed on the question of whether A and B subsets of phosphate occupy overlapping or distinct sites. To this end, it has been necessary to label the A subset with ^{32}P.

1a. Labeling of MAP_2 A site phosphates by phosphorylation in vivo (S. Tsuyama): Bilateral intraventricular injections of $^{32}P_i$ into adult rat brains has so far been the method used. Animals are sacrificed after 4 to 24 hr. Rat brain supernatant fraction is combined with carrier, in the form of twice-cycled bovine brain microtubule protein, and the mixture is at once boiled (inactivating protein kinases and phosphatases). MAP_2 is further purified from the heat stable fraction by gel filtration. The $^{32}P_i$ is much better retained in the brain than after intracerebral injection (described last year), and there is extensive turnover of phospholipid. Although MAP_1 (MW 300,000) and MAP_2 were the most conspicuously labeled proteins in the crude rat brain supernatant (contaning 8.7 and 6.7%, respectively, of the total bound ^{32}P), the overall labeling of protein was disappointingly low. Expressing the ^{32}P content as % of the total injected, the acid soluble fraction was found to contain 20 and 11% after 4 and 24 hr, respectively; corresponding amounts in phospholipid were 5.1 and 6.7%, and in protein 0.11 and 0.12%. The latter percentages were even lower when the amount of $^{32}P_i$ injected was increased, evidently because of radiation necrosis of the brain, with activation of protease which fragmented the labeled and carrier MAP_2 even during a brief period at 0°.

To ensure uniform labeling of A site phosphates, it would be desirable that the MAP_2 protein itself be newly synthesized during the period of exposure to $^{32}P_i$. The turnover time of MAP_2 in brain is very long and indeterminate. Unfortunately we have so far found cultured glial or neuronal cells to contain little if any MAP_2 even under conditions where neurite-like processes are formed (C. Patterson). Currently we are studying MAP_2 phosphorylation in brain slices, in tissue cultured from embryonic brain, and after intracranial injection in new born rats. An interesting incidental observation is that the MAP_2 phosphorylated in newborn brain appears to be heat-labile. So far these methods have not given improved results, but many variations remain to be tried.

1b. Evidence that A and B subsets of phosphate occupy different sites on MAP_2. (A. Murthy, G. Bramblett, S. Tsuyama): Differential susceptibility to phosphatase was studied using MAP_2 in which either A or B phosphates were labeled. Labeling of A sites is described above. B sites were labeled as described last year, by phosphorylation by the coassembly of cAMP-kinase; in most experiments this has given no overlapping labeling by exchange of A sites with ATP. Evidence that the introduction of 10 to 12 mol phosphate/mol MAP_2 may represent saturation of specific sites, rather than partial labeling of many sites, is provided by the observation (in collaboration with T. Singh et al.) that a casein kinase can introduce more than 60 mol per mol MAP_2. The maximum extent of phosphorylation by cAMP kinase was the same whether MAP_2 was preassembled in microtubules, or maintained disassembled with podophyllotoxin, and also with a mixture reconstituted from pure MAP_2 and kinase catalytic subunit.

The following purified protein phosphatases were used: our brain enzyme (type 1 and/or 2a catalytic subunit), smooth muscle phosphatase 1 (type 2a), calcineurin (type 2b), and SMP-2 (type 2c). Under conditions where these respectively released 84, 78, 44 and 22% of ^{32}P from MAP_2 labeled in B phosphates, no measurable release of A phosphate was observed. With some batches of MAP_2 we have recently observed a very slow release of A phosphate with both the above, and a second (section 1d) protein phosphatase purified from brain.

A second line of evidence stems from isolating projection and binding domain fragments of MAP_2, as described last year. Whereas B sites are in both domains, A sites which turned over in vivo were confined to the projection domain.

1c. Characteristics of A site phosphates (A. Murthy, C. Patterson): PSer and PThr were recovered in 6:1 ratio from partial acid hydrolysates of MAP_2 labeled in either A or B sites. Because of the phosphatase-resistant nature of the A phosphates, it seems important to establish whether all of the A phosphate is in the form of these amino acid esters. We have derivatized phosphovitin by the methylamine procedure, and shown that with our analyzer the PSer derivative elutes between His and Lys. We are now determining whether we can get quantitative recoveries with a protein (ovalbumin) having a phosphate content similar to that of MAP_2. The relative rates at which alkali catalyzes release from A and B sites will also be determined.

1d. Brain protein phosphatases active towards MAP_2 (C. Patterson): All studies have been with labeled B sites, as it has so far been impractical to prepare enough labeled A site MAP_2 to use it as a substrate for enzyme purification.

Using a phosphohistone as substrate, we had previously purified 3000 fold a phosphatase from brain soluble fraction, which comprised 2 polypeptides, by SDS-PAGE, of molecular weights 36,000 and 47,000. The procedure was similar to one which, with skeletal muscle, yields a mixture of the catalytic subunits of types 1 and 2a (P. Cohen classification). During purification the ratio of activities, MAP_2/histone, shifted from 3 in crude supernatant to 0.25, suggesting brain might contain another phosphatase with specificity for MAP_2. On storage (in 50% glycerol at -15°) all fractions of this "BPase-1" have lost 95% of their activity for histone, without loss of MAP_2 activity.

Using MAP_2 as a substrate we have now partially purified a BPase-2 by sequential column steps using DEAE-Sephacel, Sephacryl S300 and a Sepharose affinity column prepared from thiophosphorylated (B site) MAP_2. During purification the ratio of activities, MAP_2/histone, shifted from 3 to 20. By using large amounts of BPase-2 we have also recently been able to release up to 2/3 of the A site (labeled in vivo) phosphate, without detectable proteolytic digestion. It should be noted that MAP_2 is exceptionally sensitive to protease, and all combinations of inhibitors have failed to prevent digestion with commercially available phosphatases.

Assays of the initial rate of phosphate release suggest that MAP_2 (or at least a subset of its B site phosphates) is a good substrate for many phosphatases. With crude supernatant fractions the activity ratio, MAP_2/histone, was found to be 5.4 for liver, 3.5 for brain, 2.2 for heart and 1.3 for skeletal muscle. The specific activity for MAP_2 in brain was 2 x > liver, and 3 x > muscle or heart.

Rates for the above 2 substrates, and myosin light chain, were also compared using 4 pure protein phosphatases (as well as BPase-1 and -2): calcineurin, smooth muscle phosphatases 1 and 2, and MgATP-requiring phosphatase (a complex of inhibitor-2 and type 1 catalytic subunit). These include all 4 classes of protein phosphatase in the nomenclature of P. Cohen and collaborators. Histone was a poor substrate for all except BPase-1. Myosin light chain was the only effective substrate for SMP-2, and was slightly preferred over MAP_2 by SMP-1, MgATP-phosphatase, and BPase-1. Only calcineurin preferred MAP_2 to both other substrates.

2. Tubulin Tyrosinolation (G. Bramblett): This project has been limited to attempts to improve the purification of HeLa tubulin. We succeeded to self-assemble microtubules using extraction buffers in D_2O as described by J. Olmsted.

Proposed Course of Project:

1. Confirmation that A and B sites are distinct: We plan to compare the ^{32}P peptides obtained by limited protease digestion of MAP_2 labeled on A or B sites. In preliminary experiments (S. Tsuyama) trypsin has given very complex, and chymotrypsin complex but resolvable, mixtures. Papain, and especially Staph V8 protease, initially gave only a few large fragments in relatively good yield. Since MAP_2 is very large, it may be useful to first isolate such fragments, by HPLC or SDS-PAGE, and then carry out further digestion and peptide mapping.

2. Nature of the B site phosphate: These sites are unoccupied in MAP_2 as isolated. This might be because: a) they have been vacated by phosphatase action during MAP_2 purification; b) they are regulated empty under the physiological conditions of the tissue of origin; or c) their phosphorylation *in vitro* is artifactual and never occurs *in vivo*. The unexciting possibility (c) is rendered somewhat less likely by the intimate association between MAP_2 and cAMP-dependent kinase.

3. Nature of the A site phosphates: Immediate questions are whether these 10 phosphate residues are all present as amino acid esters, and whether the incorporation of $^{32}P_i$ *in vivo* labels all or most of them. At present the only approach to the second question seems to be to expose MAP_2 to labeled phosphate in cells where the protein itself is turning over.

4. Functions of MAP_2 in 4 defined states of phosphorylation: We have the states A+B+ and A+B-, and have shown that MAP_2 in these states has quite different characteristics in promoting microtubule assembly. The enzyme BPase-2 (section 1d above) may be promising for the preparation of A-B-. A-B+ might be obtained by thiophosphorylating the B sites *in vitro*, and then treating with BPase-2.

5. The physiological kinase and phosphatase for the A sites: We have phosphatase preparations from brain which do, and do not, show substrate preference for B site phosphates. The problem now is to obtain MAP_2 authentically labeled at A sites in sufficient quantity that it can be used as a substrate for enzyme purification. Meantime the colocalization of MAP_2 with calcineurin and a calmodulin kinase (the "principle post-synaptic density protein") should not be overlooked. It is pertinent to note that we find MAP_1 becomes more labeled than MAP_2 *in vivo*, but is not phosphorylated by the cAMP kinase *in vitro*.

6. Functions of A and B site phosphates: We find that B sites are about 4 times more concentrated in the binding domain than in the projection domain, consistent with the fact that these phosphates affect microtubule assembly. Since A sites are confined to the projection domain, these phosphates are candidates to modulate the interaction of microtubules, or MAP_2, with other organelles. To this end we have purified neurofilaments from bovine spinal cord, and begun to study their affinity for MAP_2 (G. Bramblett).

Are the A site phosphates "structural" in function, i.e., phosphates which do not turn over, and might be inaccessible to phosphatases because they are folded "inside" after being added to nascent polypeptide chains?

Finally the restricted localization of MAP_2 in dendrites suggests that its real functions may prove to be different from any so far inferred from its behavior in vitro.

Publications:

1. Miyatake, K. and Flavin, M.: Characteristics of tubulin aggregation by tubulin-binding proteins from brain and by synthetic polycations. Int. J. Biochem. 15:1305-1312, 1983.

2. Murthy, A. and Flavin, M.: Microtubule assembly using the microtubule-associated protein MAP-2 prepared in defined states of phosphorylation with protein kinase and phosphatase. Eur. J. Biochem. 137:37-46, 1983.

3. Nath, J. and Flavin, M.: Tubulin tyrosinolated in vivo can be different from that tyrosinolated in vitro. Biochim. Biophys. Acta 803:314-322, 1984.

4. Flavin, M. and Murofushi, H.: Tyrosine incorporation in tubulin, in "Methods in Enzymology: Post-translational modifications: (Eds. K. Moldave and F. Wold) Vol. 106, Article 22, 1984.

5. Singh, T., Akatsuka, A., Huang, K-P., Murthy, A. and Flavin, M.: Cyclic nucleotide and Ca^{2+}-independent phosphorylation of tubulin and microtubule-associated protein-2 by glycogen synthase (casein) kinase-1. Biochem. Biophys. Res. Commun. 121:19-26, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00506-09 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Acanthamoeba myosins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Other: Joseph Albanesi, Staff Fellow
Mark Atkinson, Visiting Associate
Graham Coté, Visiting Fellow
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Hisao Fujisaki, Visiting Fellow
Victor Pribluda, Guest Worker
Takashi Ueno, Guest Worker Ray Scharff, Chemist

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, NCI (Dr. Ettore Appella)
Department of Physiology, University of Connecticut (Dr. Michael Sheetz)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
8.75	8.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The amino acid sequence of a 58-residue segment of the small peptide released by chymotrypsin from the COOH-end of the heavy chains of Acanthamoeba myosin II has been determined. The first 36-residues have a sequence predicting an α-helical coiled coil conformation. The three regulatory phosphorylatable serines are at positions 46, 51 and 56 in repeating pentapeptides of nearly identical sequence. The myosin II molecule missing this small peptide is unable to form bipolar filaments although it does form parallel dimers. It has full Ca^2-ATPase activity but no actin-activated ATPase activity nor does it bind to F-actin in the presence of ATP. These and other data support the idea that only filaments of myosin II with the proper conformation (dephosphorylated) have actin-activated ATPase activity. The catalytic and regulatory domains of Acanthamoeba myosins IA and IB have been mapped. They are near each other in the NH_2-terminal halves of the heavy chains with the catalytic sites being closer to the terminus. The actin-activated ATPase activity of myosins IA and IB show an actin-dependent myosin cooperativity which may explain the complex enzyme kinetics reported previously. Single-headed myosin I is able to crosslink actin filaments suggesting that the myosins either have two actin-binding sites or, in the presence of F-actin, can form oligomers. Beads coated with myosin I show ATP-dependent movement along actin cables demonstrating that these single-headed enzymes are able to support motile activity.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives: Myosins are actin-activated ATPases that, together with actin, are responsible for the contraction of muscle and, as much more recently discovered, many motile activities of non-muscle cells. We previously discovered the presence of three myosin isoenzymes in the amoeba, Acanthamoeba castellanii, and demonstrated that they were different gene products. The actin-activated ATPase activities of all three myosins are regulated through phosphorylation of their heavy chains, a type of regulation discovered in this laboratory. Most of our recent activities are concerned with understanding the mechanism of the actin-activated ATPase activity of these enzymes, how it is regulated by heavy chain phosphorylation and the way in which the energy released by hydrolysis of ATP is converted into mechanical movement.

Methods Employed and Major Findings: Acanthamoeba myosin II. This enzyme consists of two heavy chains of Mr 185,000 and two pairs of light chains of Mr 17,500 and 17,000. It forms small bipolar filaments under physiological conditions. Previously, we reported that there are three phosphorylatable serines near the tail end of each heavy chain and that phosphorylation of these serines inactivates the actin-activated ATPase activity of the myosin. Controlled hydrolysis by chymotrypsin releases a small peptide from the ends of the two heavy chains containing all three serines. The peptide probably contains between 60 and 70 amino acids.

We have now determined the amino acid sequence of the first 58 residues of this peptide beginning at the NH_2-terminal (the chymotrypsin cleavage site). The first 36 residues have a sequence that predicts an α-helical coiled coil structure as expected for the tail of the myosin molecule. It is then interrupted by a Gly-Thr-Pro sequence at positions 38-40. The three phosphorylation sites are at positions 46, 51 and 56 in a sequence that contains a nearly identical repeating pentapeptide: Arg-Gly-Gly-Ser-Thr-Arg-Gly-Ala-Ser-Ala-Arg-Gly-Ala-Ser-Val-Arg. Thus, each serine is 3 residues after and 2 residues before an arginine. This is the first time the sequence has been determined around the regulatory site of any non-muscle myosin. The sequence differs from that for substrates of other known protein kinases.

The next question to consider is how the state of phosphorylation of residues this far from the catalytic and actin-binding sites, which occur at least 100 nm away in the head of the molecule, can regulate actin-activated ATPase activity. Last year, we suggested that regulation might occur at the level of the filament. Myosin II is always filamentous under conditions of the enzyme assay and the presence of phosphorylated molecules in copolymers with dephosphorylated molecules inactivated the otherwise active dephosphorylated molecules. Therefore, there appeared to be an active filament conformation that could be attained by dephosphorylated myosin II but not by phosphorylated myosin II and which could be disrupted by phosphorylated molecules in copolymers. These observations have now been extended and seem to be confirmed by more recent data.

Under controlled conditions, trypsin cleaves myosin II at a single site in the head region of the heavy chain forming one peptide of Mr 75,000 and another of 110,000. These peptides dissociate only under denaturing conditions, in contrast to HMM and S-1 prepared by similar proteolysis of muscle myosins. The trypsin-cleaved myosin II retains complete Ca^{2+}-ATPase activity but loses all actin-activated ATPase activity. It makes bipolar filaments that are indistinguishable from those of undigested myosin II. As observed for phosphorylated myosin II, trypsin-cleaved myosin II forms copolymers with dephosphorylated myosin II and inactivates the dephosphorylated myosin II in the copolymers. These results provide additional evidence that filaments of the right conformation are essential for actin-activated ATPase activity to be expressed by dephosphorylated myosin II. Apparently a single nick in the heavy chains is sufficient to alter this conformation.

Studies of the chymotrypsin-cleaved myosin II, the molecule remaining after removal of the tip of the tail containing the three phosphorylation sites, have been even more revealing. Electron microscopy demonstrated that the tail of the chymotrypsin-cleaved molecule was reduced from a length of 89 nm to 76 nm, about the correct amount for the loss of a peptide of 60-70 residues. The chymotrypsin-cleaved molecules were unable to form bipolar filaments but, instead, appeared to associate into parallel (monopolar) dimers. Thus, this small region at the end of the tail of the heavy chains seems to be essential for the formation of bipolar filaments but not for the formation of the parallel dimers that are thought to be an intermediate step in myosin polymerization. Of equal interest is the fact that the chymotrypsin-cleaved myosin II is unable to bind [illegible] enzyme assay conditions) althought it bind[s] [illegible] f ATP, and has no actin-activated ATPase [a]ctivity. [illegible]in II does not form copolymers with nat[ive myosin]. [illegible]ults support the idea that filaments of [myosin II] [illegible]for actin-activated ATPase activity a[re regulated] [illegible]ed physiologically by the state of phos[phorylation] [illegible]very near the end of the tail.

Acanthamoeba myos[in IA and IB] [illegible]nes IA and IB is slower because they can [be purified] [illegible]ifficulty and in smaller amounts than myos[in II]. [illegible]r, to map the catalytic and regulatory do[mains] [illegible]fragments of their heavy chains labeled at [the] [illegible]the regulatory serine by ^{32}P. The catalyt[ic site] [illegible]in 38 kDA of the NH_2-terminal end of t[he heavy chain] [illegible]norylatin site is within 40 kDA of the cat[alytic site]. The catalytic and phosphorylation sites of myosin IB are within 10 kDA of each other and both are within 60 kDA of the NH_2-terminus of the 125 kDA heavy chain, the catalytic site being closer to th NH_2-end.

Section 4

The kinetics of the actin-activated ATPase activity of myosins IA and IB are very complex. As the actin concentration is increased, the ATPase activity first increases, as expected, but then decreases followed by another activation phase as the actin concentration is further increased. Some insights were gained into this phenomenon when it was found that the specific activity of the actin-activated ATPase depends on the myosin concentration.

There is a cooperative increase in specific activity, at a constant actin concentration, as the myosin concentration is increased. At higher actin concentrations, it requires higher myosin concentrations for this cooperativity to be expressed so the myosin-myosin interaction is actin-dependent. The mechanism of this cooperativity is not yet understood.

Myosin IA and IB appear to be unable to form bipolar filaments, the structures that are generally involved in actomyosin-dependent motility. We have found, however, that, as measured both by light scattering and by low-shear viscosity, both myosin IA and IB appear to be able to crosslink filaments of F-actin. Myosin IA and IB have much greater effect, for example, than single-headed muscle myosin subfragment-1 and are at least as effective as two-headed muscle heavy meromyosin. As myosins IA and IB are both single-headed molecules, their abilities to crosslink actin filements imply either that they are able to form dimers or other oligomers in the presence of F-actin or that each myosin I molecule contains two binding sites for F-actin. Either property could explain the actin-crosslinking ability of myosin I but the inability to detect myosin I oligomers in the absence of F-actin. Either property could also explain the actin-activated myosin I ATPase activity. Crosslinking of actin filaments by either mechanism would allow myosin I to function in motile activities by the classical sliding filament model.

In collaboration with Dr. Michael Sheetz, we have found that particles coated with myosin I bind to and move along actin cables. Movement depends on the presence of ATP, occurs only when the myosin I heavy chains are phosphorylated (only phosphorylated myosin I has actin-activated ATPase activity) and is inhibited by antibodies to myosin I that inhibit actin-activated ATPase activity. Thus, these experiments provide direct evidence for the ability of myosin I to function in a motile process. We do not know if the myosin on the beads is in the form of oligomers or if monomers adsorbed to the surface are sufficient to move the particles. These observations may provide experimental support for our earlier speculation that myosin I isoenzymes might function by virtue of association with vesicle or other membranes rather than as filaments.

Proposed Course of Research:

Myosin II: We will attempt to isolate the peptide containing the catalytic site and determine its sequence and relative position in the heavy chain. We will continue our efforts to purify the heavy chain kinase. With the kinase and the synthetic 20-residue peptide containing the phosphorylation sites, we will study the relative specificity for the three sites and compare the activity of the myosin heavy chain kinase to other kinases. Further efforts will be made to correlate actin-activated ATPase activity with filament formation.

Myosin I: Efforts will be continued to understand the molecular basis of the myosin cooperativity and the crosslinking of actin filaments by myosin. Additional physical studies will be carried out to determine the shape of the myosin I molecules. We will attempt to isolate small peptides containing the phosphorylation and catalytic sites to determine their sequences and compare them to the sequences of the corresponding regions of myosin II and of other myosins. We will prepare cytoskeletal complexes in an effort to ascertain the nature of the physiological complexes of actin with myosins I and myosin II.

Publications:

Hammer, J.A. III, Albanesi, J.P., and Korn, E.D.: Purification and characterization of a myosin I heavy chain kinase from Acanthamoeba castellanii. J. Biol. Chem. 258:10168-10175, 1983.

Albanesi, J.P., Hammer, J.A. III and Korn, E.D.: The interaction of F-actin with phosphorylated and unphosphorylated myosins IA and IB from Acanthamoeba castellanii. J. Biol. Chem. 258:10176-10181, 1983.

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Kuznicki, J. and Korn, E.D.: Interdependence of factors affecting the actin-activated ATPase activity of myosin II from Acanthamoeba castellanii. J. Biol. Chem. In Press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00508-03 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation and Characterization of Acanthamoeba Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Blair Bowers	Research Biologist	LCB, NHLBI
Others:	Thomas C. Hohman	Staff Fellow	LCB, NHLBI
	Thomas E. Olszewski	Biologist	LCB, NHLBI
	Kyung Nam	Biologist	LCB, NHLBI
	William Egan	Acting Chief, Biophysics Branch	Natl. Center for Drugs and Biologics

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.7	0.6	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to elucidate mechanisms of membrane recycling in endocytic cells, we have isolated highly purified surface membranes and phagosome membrane fractions to compare their chemical composition. Phosphorus NMR was used to examine the ratio of phospholipids to glycolipid (containing phosphono groups) in the membrane fractions. No differences between the fractions were found in the phosphorus-containing components or in the ratio of phosphono groups to phosphodiester groups. In contrast, the peptide composition of the fractions appears to be significantly different. Only about one-third of the major Coomassie Blue staining bands in one dimensional polyacrylamide gels have the same mobility. The purity of the fractions and analyses of likely sources of contaminating proteins rules out that the differences seen can be attributed entirely to contaminating material. The origin of the peptide differences is being explored.

Project Description:

Objectives: Our objectives are to compare the chemical composition of Acanthamoeba plasma membrane with that of phagolysosome (vacuolar) membrane. Despite an apparent rapid exchange of membrane between the cell surface reported previously, structural differences between these two membranes suggest the possibility of chemical differences. Specific information as to the nature and extent of chemical differences that may exist between the two membrane compartments is essential to understanding the process of membrane recycling.

Methods Employed: Radioactive tracer methods were used to assess purity of the two membrane fractions. Iodination of the intact cell surface provided a marker for plasma membrane isolation, and cells grown in 35-S methionine were used to monitor contamination of phagolysosome membranes isolated from nonradioactive cells. Membranes were isolated by density gradient centrifugation: on Percoll gradients for plasma membrane and on sucrose gradients for phagosome membranes. Polyacrylamide gel electrophoresis and autoradiography were used to study the protein composition of the purified membrane fractions. Standard biochemical procedures were used for protein and enzymatic determinations. Light and electron microscopy were used to monitor the isolation procedure and to determine the purity of the final product. P-NMR studies were performed to measure phospholipid/glycolipid ratios.

Major Findings: As described previously, we have obtained 2 highly purified membrane fractions from Acanthamoeba: the plasma membrane (PM) with a 42-fold purification (estimated to be 85-90% pure) and phagosome membrane (PhM), estimated to be 90-95% pure.

1. In collaboration with William Egan, National Center for Drugs and Biologics; we have examined the two fractions by ^{31}P NMR spectroscopy to determine whether the ratio of phospholipid/glycolipid is the same in the two membrane fractions. The NMR spectra were essentially identical, showing two major phosphodiester peaks and five phosphono peaks. The phosphono compounds are components of lipophosphonoglycan, a glycolipid comprising approximately one-third the mass of the membrane, and the phosphodiesters are found in the glycolipid as well as phospholipids of the membranes. The ratio of the sums of the phosphono/phosphodiester peaks were the same in both membranes, suggesting that the ratio of phopholipid to glycolipid in the isolated fractions is identical. Attempts to compare the ratio of protein to total phospholipid plus glycolipid have not so far yielded reproducible results, because of variability especially in protein analyses.

2. The peptide content of the two fractions is markedly different. Judged by one-dimensional polyacrylamide gel electrophoresis, only about one-third of the Coomassie Blue staining bands have the same mobility in both fractions. The same band patterns for each membrane is obtained consistently in different isolations. Both isolations are carried out in the presence of protease inhibitors.

Actin isolates with PM fractions and not with PhM fractions. Actin, easily identified on polyacrylamide gels, accounts for about 10% of the total protein

of the plasma membranes and thus appears to be the major contaminant of that fraction. The two fractions are estimated to be 5-10% contaminated from other sources. We have identified one of these as contractile vacuole membrane, which is morphologically identifiable in electron micrographs. The enzymatic marker for this membrane, alkaline phosphatase, is also enriched in both fractions over the cell homogenate. Given that the fractions are relatively pure and that a significant portion of the contamination for both membranes is the same (and thus should not contribute to differences we observed), the peptide differences appear to reflect real differences in the membrane fractions.

3. In order to explore further the origin of protein differences between the 2 membrane fractions we have examined the fate of radiolabeled surface peptides after internalization.

Cells were surface iodinated and the labeled species compared in aliquots of whole cells, in isolated plasma membrane and in isolated phagosome membrane (from surface labeled cells allowed to take up particles). Autoradiograms of polyacrylamide gels from the 3 preparations were virtually identical. The radiolabeled species comprise about 8 bands. None correspond to major Coomassie blue staining bands. Four of the eight are consistently more heavily iodinated than the others. These results suggest that at least 8 iodinatable peptides are internalized in phagosomes in the same proportion found in the plasma membrane. We have also looked for evidence of degradation of the iodinated peptides with reincubation of cells after surface labeling. Labeled cells were reincubated and sampled after 2, 4, 6 and 10 hours. No clear evidence of change was observed until 4 hours. At that point a 160 K peptide showed a shift in mobility and had essentially disappeared after 10 hours. There appeared to be a roughly corresponding loss of the 160 K peptide and decreases in a 85 K peptide with increases in lower molecular weight bands.

The increase in mobility of iodinatable species could be due to clipping of carbohydrate chains by lysosomal glycosidases as the membranes circulate through the vacuolar system. We attempted to test this hypothesis by incubating surface labeled cells in concentrated lysosomal enzymes for up to 60 min at 30° at pH 4.5. This maneuver did not result in any shift of radiolabeled bands.

Significance to Biomedical Research: Several protective cells of the body such as macrophages, perform their function by the process of endocytosis. Knowledge of the events of this process will aid in treating or preventing diseases associated with a breakdown or alteration of normal endocytic events.

Proposed Course of Research: We will continue to isolate membranes in order to obtain sufficient quantities for additional analysis of the compositional differences between the membranes. These analyses will include determination of the protein/phospholipid ratio and the protein/sterol ratio. We will examine origins of the peptide differences between membrane fractions.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00509-03 LCB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lysosomes and Hydrolase Secretion in Acanthamoeba

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Thomas C. Hohman	Staff Fellow -	LCB, NHLBI
	Blair Bowers	Research Biologist	LCB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Acanthamoeba continuously releases lysosomal hydrolases into its growth medium. This release is specific for lysosomal hydrolases and is energy dependent. Hydrolases secreted by pinocytosing cells separate into two classes: one released at about 17% of the cellular content per hour and the other at about 4% per hour. We have suggested that hydrolase secretion is a consequence of membrane returning to the cell surface from digestive vacuoles via shuttle vesicles. When the vesicles fragment from the acidic digestive vacuoles, small amounts of the hydrolases are trapped in the vesicle lumen. Some of the hydrolases that have a higher affinity for membranes are bound to the vesicle membrane as well as being trapped in the lumen. When shuttle vesicles fuse with the plasma membrane the luminal contents are released and the vesicle membrane now exposed to the growth medium experiences a significant pH change. This change in pH decreases the affinity of hydrolases for membranes and causes the membrane-bound hydrolases to be unloaded. Hydrolases trapped in the shuttle vesicle lumen are released at low rates and form one of the classes of hydrolases. Hydrolases bound to the vesicle membranes as well as being trapped in the lumen are released at higher rates and form the second class of hydrolases. This model is supported by recent data. The pattern of differential hydrolase secretion, characteristic of pinocytosing amoebae, is lost when cells are incubated in growth media containing chloroquine or ammonium acetate, agents which raise the intravacuolar pH. Additionally, this pattern of hydrolase secretion is also lost by amoebae that have become saturated with yeast. This latter change in hydrolase secretion occurs concomitantly with an increase in vacuolar pH. All of these observations are consistent with our hypothesis that vacuolar pH is a factor regulating hydrolase secretion.

Project Description:

Objectives: Using Acanthamoeba as a model system, our long-range objectives are to understand the mechanisms involved in the exchange between internal membrane systems and the plasma membrane. The kinetics and physiology of lysosome enzyme secretion is one window on membrane exchange since the hydrolases clearly enter the cell's digestive compartment and are also released into the external medium. Thus, it is likely that they serve as a "marker" for membrane movement. This year the major thrust has been toward characterizing the mechanisms regulating the release of lysosomal enzymes under several experimental conditions.

Methods Employed: Fluorometric and colorimetric assays were used for enzyme assays. Molecular probes that have pH dependent fluorescence and can only enter the vacuolar compartment of the amoeba cell, were used to estimate vacuolar pH under several physiological conditions. Other standard biochemical or physiological procedures were utilized as necessary for the experiments.

Major Findings:

(1) Incorporation of Weak Bases into Amoeba Growth Medium Changes the Pattern of Hydrolase Secretion.

Our model for hydrolase secretion predicts that increasing the pH of digestive vacuoles will decrease the affinity of hydrolase for vacuolar membranes and will decrease the rate at which membrane-bound hydrolases are secreted. Changes in vacuolar pH are not expected to effect the rate at which soluble hydrolases are secreted. To test these predictions amoebae were incubated in growth medium supplemented with 10 mM ammonium acetate or 1 mM chloroquine. Both of these agents are known to accumulate in the vacuolar compartment and to raise vacuolar pH. Under these conditions acid phosphatase, a soluble lysosomal hydrolase, was released at about 3% of the cellular content per hour, a rate similar to that observed with cells incubated in normal medium. Hexosaminidase, a representative membrane-bound hydrolase, was secreted at about 4% per hour. Pinocytosing amoebae in normal growth medium secrete hexosaminidase activity at about 18% per hour. These results are clearly consistent with our model, if ammonium acetate and chloroquine added to amoeba growth medium increase the pH of the amoeba vacuolar compartment.

(2) Estimates of Vacuolar pH in Pinocytosing Amoebae

Fluorescein isothiocyanate can be covalently crosslinked to dextran or B-lactoglobulin to produce labels that have pH dependent fluorescence and that enter amoeba only via endocytosis. Once these labels have been engulfed by amoeba, their fluorescence can be used to estimate vacuolar pH. With this technique we measured the vacuolar pH of pinocytosing amoebae incubated in normal growth medium and incubated in medium containing 10 mM ammonium acetate or 1 mM chloroquine. Cells incubated in normal growth medium have a vacuolar pH of about 4.8. Cells incubated in media supplemented with 1 mM chloroquine or 10 mM ammonium acetate, however, have a vacuolar pH of about 6.8. Adding ammonium acetate or chloroquine to growth medium increases vacuolar pH to that of the growth medium.

(3) Hydrolase Secretion by Amoebae Phagocytosing Yeast

Studies of hydrolase secretion in other cell systems have shown that phagocytosis of yeast and some other particle types stimulates hydrolase secretion. We studied hydrolase secretion by amoebae that were phagocytosing yeast to determine whether the secretion kinetics of either or both classes of hydrolases were stimulated by particle phagocytosis. We observed that the secretion of the soluble hydrolases, those that do not bind to vacuolar membranes, increased from about 3% of the cellular content per hour to about 10% per hour when the amoebae became saturated with yeast. Secretion of the membrane-bound hydrolases, however, decreased from about 17% per hour to about 11% per hour. Cells that were saturated with yeast lost the differential pattern of hydrolase secretion. Our results with ammonium acetate and chloroquine suggested that phagocytosis of yeast may also alter vacuolar pH.

(4) Estimate of Vacuolar pH in Phagocytosing Cells

To estimate the vacuolar pH of phagocytosing amoebae we coupled fluorescein isothiocyanate to the surface of yeast and measured the fluorescence of these yeast after they were ingested. From these measurements we estimate the vacuolar pH of phagocytosing yeast to be about 6.0. This value is significantly different from that observed in pinocytosing amoebae and can account for the lost of the differential pattern of hydrolase secretion that accompanies phagocytosis of yeast.

(5) Hydrolase Secretion by Amoebae Phagocytosing Yeast in Medium Supplemented with Ammonium Acetate.

If the changes in hydrolase secretion caused by ammonium acetate and phagocytosis of yeast occur by the same mechanism, we would not expect ammonium acetate to affect the secretion pattern of amoebae saturated with yeast. To test this expectation amoeba were incubated in either normal growth medium or growth medium supplemented with 10 mM ammonium acetate for 30 minutes. Previous experiments with fluorescent labels demonstrated that with this length of incubation ammonium acetate effectively increases vacuolar pH to that of the growth medium. After this incubation the amoeba were allowed to phagocytose yeast and hydrolase secretion was measured. Under these conditions acid phosphatase and hexosaminidase activities were released at about 10% of the cellular content per hour by cells treated with or without ammonium acetate.

Significance to Biomedical Research:

Lysosomal dysfunction has been implicated in a large number of mammalian diseases including genetic disorders as well as parasitic infections. Understanding normal lysosomal functioning and cellular traffic between lysosomes and the cell exterior will provide insight into more effective ways of treating parasitic diseases and lysosomal dysfunctions.

Proposed Course of the Project:

Having characterized the secretion patterns and the pH regulation of hydrolase secretion in Acanthamoeba, the next effort will be to isolate and purify several of the lysosomal hydrolases and to prepare antibody against the

purified enzymes. Available antibody will allow us to characterize the steps involved in hydrolase processing. Data from other systems suggests that secreted hydrolases may be distinct from the isoenzymes retained inside the cells. If the hydrolases in Acanthamoeba conform to this pattern then those secreted may be a marker for shuttle vesicles which to date have eluded efforts to study them.

Publications:

T.C. Hohman and B. Bowers. 1984. Hydrolase Secretion is a Consequence of Membrane Recycling. J. Cell Biol. 98: 246-252.

T.C. Hohman and B. Bowers. 1984. Vacuolar pH is a Factor Regulating Hydrolase Secretion. (to be submitted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00510-03 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The effect of troponin-tropomyosin on the interaction of myosin with actin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Lois E. Greene	Research Chemist	LCB, NHLBI
Others:	Joseph M. Chalovich	Staff Fellow	LCB, NHLBI
	Roger Craig	Assistant Professor of Anatomy	Univ. of Mass. Med. School

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on the binding of S-1 to the troponin-tropomyosin-actin complex suggest that S-1 can bind to actin in at least two different conformations. There is a conformation that binds weakly to actin at an angle postulated to be about 90° and a conformation that binds strongly to actin at about a 45° angle. The former conformation only occurs transiently in the cross-bridge cycle when ATP or ADP·Pi is bound to myosin, while the latter conformation occurs in the presence of ADP or absence of nucleotide. A stable analog of the 90° weak-binding conformation was obtained by modifying S-1 with the bifunctional thiol reagent, N-N'-p-phenylendimaleimide. In the presence of ATP or PPi, pPDM·S-1 binds to actin as weakly as does S-1 + ATP and this binding is unaffected by troponin-tropomyosin. In the presence of ADP or absence of nucleotide, there is a small, but significant difference between the binding of pPDM·S-1 and the binding of S-1 + ATP to actin. This indicates that by artificially modifying the active site of S-1, the myosin cross-bridge may exist in more than just the two normally occurring conformational states. This may be related to the cross-bridge undergoing a continual rotation on the actin as it changes from the 90° to the 45° conformation during the cross-bridge cycle. In addition, studies using S-1 cross-linked to actin by the zero length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide, indicate that even though the myosin is cross-linked to actin, it is still able to oscillate between the 90° and 45° conformations during the actomyosin ATPase cycle. Electron micrographs of the cross-linked actin·S-1 preparation show that the structure of the cross-linked S-1 is very different in the presence and absence of ATP. This provides structural evidence for two different conformations.

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Project Description:

Objectives: The myosin cross-bridge is thought to oscillate between two major conformational states during the cross-bridge cycle. In one state, which occurs in the absence of ATP, myosin binds very tightly to actin at about a 45° angle. In the second conformational state, which occurs only transiently when ATP or ADP·Pi is bound to myosin, myosin binds weakly to actin at an angle postulated to be 90°. These two conformations also differ in that troponin-tropomyosin markedly inhibits the binding of S-1 to actin when it is in the 45° state, but has no significant effect when in the 90° state. In this study, we wanted to obtain a stable analog of the 90° weak-binding conformation in the absence of ATP in order to study its interaction with actin, nucleotide, and troponin-tropomyosin, as well as its structure under equilibrium conditions. We also wanted to examine the structure of S-1 and actin in the presence of ATP, by using S-1 cross-linked to actin by the zero length cross-linker, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide. In addition, we wanted to examine the effect of troponin-tropomyosin on the cross-linked actin-S-1 preparation to determine whether the cross-linked complex can undergo the oscillation between the two conformational states, as would be predicted by our model since the cross-linked complex has an ATPase activity comparable to that of non-cross-linked acto·S-1.

Methods Employed and Major Findings: The interaction of actin with S-1, modified by having its two reactive cysteines cross-linked by N-N'-p-phenylenedimaleimide (pPDM), was examined in the presence of different nucleotides. pPDM·S-1 initially has about 70% of its active sites occupied by ADP, which was trapped at the active site during the cross-linking procedure. We now find that in the presence of actin, nucleotide is rapidly exchanged at the active site of pPDM·S-1. Therefore, we were able to study the binding of different pPDM-S-1·nucleotide complexes to actin. In the presence of ATP, the binding of pPDM·S-1 to actin is virtually identical to that of unmodified S-1 in the presence of ATP. Specifically, at μ = 18 mM, 25°, pPDM·S-1 + ATP binds to unregulated actin with the same affinity as does S-1 + ATP and this binding is unaffected by troponin-tropomyosin. Similar results were obtained for the binding of of pPDM·S-1 to actin in the presence of PPi. Therefore, the conformation induced by the binding of ATP or PPi to pPDM·S-1 appears to be the same as that obtained with the transient S-1·ATP and S-1·ADP·Pi complexes. In the presence of ADP or absence of nucleotide (nucleotide removed from acto·pPDM·S-1 by Dowex treatment), there is a small, but significant difference between the binding of pPDM-S-1 to actin and the binding of S-1 + ATP. pPDM·S-1 alone and pPDM-S-1·ADP both bind about 2-3 fold stronger to unregulated actin than does S-1 + ATP. In addition, troponin-tropomyosin confers slight cooperative strengthening (2-3 fold) on the binding of pPDM·S-1 and pPDM·S-1·ADP to actin. This binding to regulated actin is only slightly affected by Ca^{2+}. These results suggest that the nucleotide bound to the active site of pPDM·S-1 causes conformational changes in the protein which in turn subtly alters its interaction with actin.

We also investigated whether S-1, which has been cross-linked to actin by the zero length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide, is able to oscillate between the 45° strong-binding conformation and the 90° weak-binding conformation as predicted by our model. Since troponin-tropomyo-

sin has very different effects on the 45° and 90° conformation, we investigated the properties of the regulated actin-S-1 cross-linked preparation. When the ratio of S-1 to actin was one to five in the cross-linked preparation, the binding studies indicate that in the presence of ADP, the cross-linked S-1 shifts the tropomyosin on the actin filament. On the other hand, the ATPase studies indicates that in the presence of ATP, the tropomyosin remains in the inhibitory position. Therefore, the position of the tropomyosin on the regulated cross-linked actin-S-1 filament is different depending on the nucleotide bound to the cross-linked S-1. This indicates that even though the S-1 is cross-linked to actin, it still is able to undergo the change from the 45° to the 90° conformation during the hydrolysis of ATP.

The structure of the cross-linked actin·S-1 preparation was also examined in the presence and absence of ATP by electron microscopy. The cross-linked actin-S-1 preparation went through several cycles of actin depolymerization followed by centrifugation, which increased the ratio of S-1 to actin in the cross-linked preparation from one to five to one to two. Electron micrographs of this cross-linked preparation show that in the absence of nucleotides, the decorated actin filament has the traditional arrowhead structure. On the other hand, in the presence of ATP, the cross-bridges no longer appear ordered, but rather S-1 appears to be attached to the decorated actin filament at variable angles, centered at about 90°. This is structural evidence that the conformation of the attached cross-bridge is different in the presence and absence of ATP. In addition, the stoichiometry of S-1 to actin in the cross-linked preparation was established as one S-1 cross-linked to one F-actin monomer.

We have also found that pPDM·S-1 can be cross-linked to actin. The pPDM·S-1-actin cross-linked complex appears to be both structurally and biochemically different from the unmodified S-1-actin cross-linked complex. On SDS gels, the pPDM·S-1-actin cross-linked complex shows a different band pattern. In addition, the pPDM·S-1-actin cross-linked complex has less of an effect on the binding of S-1 to regulated actin and a stronger affinity for nucleotide.

Significance to Biomedical Research: A large number of studies have suggested that the same basic mechanism of contractility is found in a wide variety of different systems. Processes as divergent as cell division, cytoplasmic streaming, and clot retraction on the cellular level may be driven by the same basic mechanism that drives the contraction of skeletal muscle, vascular smooth muscle and cardiac muscle on the organ level. Since this contractile system is so wide-spread, it is of major importance to understand the molecular basis for its operation and regulation.

Proposed Research: We will continue to study the structure by electron microscopy of the cross-linked actin·S-1 complex to determine the effect of different ATP analogs on its structure. We will also examine the structure of the cross-linked pPDM·S-1-actin complex. These structural studies will look for conformations intermediate between the 45° and 90° conformations. In addition, the effect of pPDM·S-1 on the actin-activated ATPase activity will be measured to determine whether the turning on of the ATPase activity by pPDM·S-1 is correlated with the ability of pPDM·S-1 to shift the tropomyosin from the weak

binding position to the strong binding position. We will also modify the two-headed fragment of myosin, HMM, with pPDM and measure its binding to actin both in the presence and absence of troponin-tropomyosin. This is to determine whether pPDM-HMM resembles HMM + ATP in its interaction with actin.

Publications:

1. Chalovich, J.M., Greene, L.E., and Eisenberg, E.: Crosslinked myosin subfragment-1: A stable analogue of the ATP complex of subfragment-1. Proc. Natl. Acad. Sci., USA, 80, 4909-4913, 1983

2. Greene, L.E.: Stoichiometry of acto·S-1 cross-linked complex. J. Biol. Chem., in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00514-01 LCB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Structure and Sequence of Non-Muscle Myosin Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John A. Hammer, III	Staff Fellow	LCB, NHLBI
	Edward D. Korn	Chief, Lab of Cell Biology	LCB, NHLBI
	Bruce M. Paterson	Research Chemist	LB, NCI

COOPERATING UNITS *(if any)*
Laboratory of Biochemistry, NCI, NIH (Bruce M. Paterson)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to elucidate the gene structure and sequence of a non-muscle myosin. This project is part of the general effort in the Lab of Cell Biology to understand the organization and function of the cytoskeleton, using as a model system the soil amoeba Acanthamoeba. Acanthamoeba expresses simultaneously at least three distinct myosin enzymes, myosin IA, myosin IB and myosin II. Using molecular cloning techniques, we have isolated and purified a myosin II heavy chain gene and a myosin IB heavy chain gene. These genes were detected in a genomic library of Acanthamoeba DNA using a heterologous myosin gene (C. elegans) as a probe. The amoeba genomic clones were identified as myosin heavy chain genes by hybrid selection of myosin messenger RNA, in vitro translation, and specific immunoprecipitation. We are currently seeking to define the basic organization of these genes and to obtain sequence data from regions of the myosins which should be highly conserved. We intend to sequence these genes in their entirety.

The significance of this work is that it will provide for the first time the sequence of a non-muscle myosin. While non-muscle and muscle myosins share many common features, non-muscle myosins do possess unique structural, enzymatic, and regulatory properties. The amoeba myosin sequence data will be of great value in furthering our understanding of the unique structural and functional aspects of the amoeba myosins. From the sequence data we will synthesize peptides, generate antibodies against these peptides, and use these antibodies as tools to analyze the function of the amoeba myosins in in vitro motility assays. We will then use these techniques to generate cytoskeletal proteins (myosins and/or other important cytoskeletal proteins) which have altered primary structure as a tool for dissecting the interrelationship between structure and function at the protein level.

Project Description:

Objectives:

(i) Immediate: Isolate the heavy chain genes for the three Acanthamoeba myosin enzymes; sequence these genes in their entirety; analyze the basic organization of these genes (eg. intron/exon pattern, structure of upstream regulatory sequences, relatedness of the 3 myosin genes in terms of their evolution).

(ii) Near Future: From the sequence data, synthesize peptides from important areas of myosin structure, generate antibodies against their peptides, and use these antibodies as tools to analyze the function of the amoeba myosins in the recently developed in vitro motility assays.

(iii) Long Range: Use molecular cloning techniques to generate cytoskeletal proteins (myosins and/or other important cytoskeletal proteins) which have altered amino acid sequences as a tool for dissecting the interrelationship between structure and function at the protein level. This approach will make use of site directed mutagenesis and cloning in prokaryotic and/or eukaryotic expression vectors, as well as a large number of biochemical and biophysical techniques which will be used to analyze the structure and function of the altered proteins.

Methods Employed: Protein purification, antibody production and analysis by Western blot, purification of DNA and RNA, in vitro translation, immunoprecipitation, analysis of DNA fragments and RNA by restriction mapping and nucleic acid hybridization (Southern and Northern blots), cloning in plasmid and bacteriophage vectors, construction of genomic libraries, hybrid selection analysis of cloned DNA, preparation and use of synthetic oligonucleotides, peptide synthesis.

Major Findings: (i) Translation of amoeba messenger RNA in an in vitro rabbit reticulocyte lysate in the presence of radioactive methionine followed by immunoprecipitation with antibodies to the amoeba myosins results in the specific immunoprecipitation of radioactive polypeptides whose heavy chain molecular weights are identical to those of the purified protein. This result strongly supports our conclusion that the 3 amoeba myosins are separate gene products and that purified myosin IA and IB, despite possessing small heavy chains for myosins, are truly representative of the molecules in vivo. (ii) A heterologous myosin gene was used to isolate an Acanthamoeba myosin II heavy chain gene and an Acanthamoeba myosin IB heavy chain gene. Initially, we found that a 2.7 kb Bam H1 fragment from the nematode C. elegans myosin heavy chain gene, which contains the actin and ATP binding sites and the active thiols, hybridized to a number of discrete DNA fragments in Southern blots of restricted amoeba DNA and to two large RNA species (5300 bp and 4250 bp) in Northern blots of amoeba messenger RNA. Therefore, this heterologous probe was used to screen a genomic library of Acanthamoeba DNA constructed in phage lambda 2001. Approximately 4 positive phage were detected per genome equivalent. Restriction mapping of 16 purified positive phage yielded 3 non-overlapping groups of phage. A single representative phage from each group was chosen for further study. One of these phage was identified as containing an amoeba myosin II heavy chain gene based on the following observations: the phage

DNA insert hybrid selected a messenger RNA which translated in vitro to yield a 185 kDa protein: the 185 kDa protein was quantitatively and specifically immunoprecipitated by myosin II antiserum; the 185 kDa protein co-migrated in SDS-PAGE with the authentic, purified myosin II heavy chain; and the phage DNA insert hybridized to the 5300 bp messenger RNA Northern blots of amoeba RNA. We are currently analyzing restriction maps of this phage with several myosin specific DNA probes. Preliminary results indicate that most or all of the myosin II heavy chain gene is present within a 6.5 kb restriction fragment. Finally, a single phage clone which does not fall within the above 3 groups and which hybrid selects a message for a 125 kDa protein and which hybridizes to the 4250 bp message in the amoeba RNA blot, we have tentatively been identified as a myosin IB heavy chain gene.

Significance to Biomedical Research: The purpose of this project is to elucidate the gene structure and sequence of a non-muscle myosin. This project is part of the general effort in the Lab of Cell Biology to understand the organization and function of the cytoskeleton, using as a model system the soil amoeba Acanthamoeba. The significance of this work is that it will provide for the first time the sequence of a non-muscle myosin. While non-muscle and muscle myosins share many common features, non-muscle myosins do possess unique structural, enzymatic, and regulatory properties. The amoeba myosin sequence data will be of great value in furthering our understanding of the unique structural and functional aspects of the amoeba myosins. Furthermore, this project is a starting point for our long range effort to use the techniques of molecular cloning to analyze cytoskeletal protein structure and function at the molecular level.

Proposed Course of Research: Continue to pursue the objectives outlined above.

Publications:

1. Hammer, J.A. III, Korn, E.D. and Paterson, B.M.: Acanthamoeba Myosin IA, IB and II Heavy Chains are Synthesized In Vitro from Acanthamoeba Messenger RNA. J. Biol. Chem. (in press)

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1983 through September 30, 1984

Research in the Laboratory of Cellular Metabolism continues to be largely concentrated on the enzymes responsible for the synthesis and degradation of cAMP and cGMP through which many hormones, drugs, and other agents influence cellular function. In addition, work is continuing on the ADP-ribosyltransferases of animal cells in an attempt to identify their physiological substrates and functions.

1. Cyclic Nucleotide Phosphodiesterases

Last year, we reported some complex effects of divalent cations, lipids and phosphodiesterase inhibitors on activity of a so-called cGMP-stimulated phosphodiesterase purified to homogeneity from calf liver. Three competitive inhibitors, papaverine, dipyridamole, and isobutylmethylxanthine (IBMX), were also able to mimic substrate and increase catalytic activity, whereas another, cilostamide, did not. Continuing data analysis using a simple interaction model for allosteric enzymes should provide a better understanding of regulatory properties of this phosphodiesterase. Finding that IBMX was a much more potent inhibitor than theophylline (1,3 dimethylxanthine), we investigated several xanthines differing in substitutents at the 1, 3, and 7 or 8 positions. All were competitive inhibitors and increased enzyme activity at low substrate concentrations. Addition of a methyl at position 7 decreased inhibitory potency of 1,3-substituted compounds. Derivatives with methyl groups in the 1 and/or 3 positions were less potent than analogous compounds with propyl or isobutyl moieties, suggesting that hydrophobic interactions may be important for inhibition by these xanthines. From a fraction obtained during purification of the cGMP-stimulated enzymes, two "low K_m" cAMP phosphodiesterases were separated. One (M_r ~ 85,000) was very sensitive to inhibition by cGMP and cilostamide. The other (M_r ~ 174,000) was relatively insensitive to cGMP and cilostamide but sensitive to Ro 20-1724. Such relatively specific inhibitors may be useful for evaluating role of individual phosphodiesterases in regulation of intracellular cAMP content or cAMP-dependent processes and were used for this purpose in experiments with 3T3-L1 adipocytes (see below).

This year we began investigation of the regulatory properties of the light-activated cGMP phosphodiesterase from retinal rod outer segments. (Other components of this system are being studied in relation to adenylate cyclase.) The purified phosphodiesterase (M_r ~ 196,000) has subunits of 88, 86 and 10 kDa. Its activity was increased > 10-fold by proteolysis with trypsin or chymotrypsin. ADP-ribosylation (of both large and small subunits) with purified ADP-ribosyltransferase A (see below) and NAD also activated but not to the extent achieved with trypsin. Although the phosphodiesterase in rod outer segment membranes was similarly activated by the transferase, the role of ADP-ribosylation in this system remains to be determined.

Study of factors involved in physiological regulation of phosphodiesterases is continuing in cultured 3T3-L1 cells which differentiate to develop phenotypic characteristics of mature adipocytes. Differentiation of confluent cells is accelerated by insulin, dexamethasone, and IBMX. As IBMX, in addition to inhibiting phosphodiesterases, can bind to adenosine receptors, compounds that act more specifically were tested for ability to replace IBMX. Adenosine receptor ligands

(agonist and antagonist) were ineffective as was cilostamide, which inhibits particulate but not soluble cAMP phosphodiesterase in these cells. Ro 20-1724, however, a selective inhibitor of soluble activity, was as effective as IBMX, suggesting that inhibition of soluble phosphodiesterase may be important in enhancing differentiation in the presence of insulin and dexamethasone. Comparison of effects of these three inhibitors on differentiated adipocytes with and without isoproterenol stimulation supports the view that, although both particulate and soluble phosphodiesterases influence cAMP content, the particulate enzyme may be more important in degradation of cAMP involved in control of lipolysis. Since combinations of Ro 20-1724 and cilostamide were not as effective as IBMX in increasing cAMP, perhaps the calmodulin-activated phosphodiesterase, which is selectively inhibited by IBMX, contributes significantly to regulation of total adipocyte cAMP content.

When differentiated adipocytes were maintained for two weeks in medium with hypothyroid, hyperthyroid, or normal concentrations of thyroid hormone, the hypothyroid cells had increased soluble and particulate cAMP phosphodiesterase activities and decreased sensitivity to isoproterenol assessed by adenylate cyclase activation or cAMP accumulation and stimulation of lipolysis in intact cells. Conversely, in hyperthyroid cells phosphodiesterase activities were decreased while sensitivity of adenylate cyclase, cell cAMP content, and lipolysis to isoproterenol was increased. No alterations in number or affinity of β-adrenergic receptors were detected. It appears that effects of thyroid status on hormone-stimulated lipolysis in these cells may be secondary to changes in both phosphodiesterase activity and receptor-adenylate cyclase coupling.

Insulin and lipolytic hormones both cause a rapid increase in particulate low K_m cAMP phosphodiesterase activity of 3T3-L1 adipocytes. The effect of insulin but not that of isoproterenol was prevented by prior treatment of cells with pertussis toxin, providing direct evidence for different mechanisms of activation by the two agents. In many cells, pertussis toxin interferes with hormonal inhibition of adenylate cyclase by catalyzing the ADP-ribosylation of a 41 kDa subunit of the inhibitory GTP-binding protein. The relationship of this protein, which was also ADP-ribosylated in the adipocytes, to insulin activation of phosphodiesterase is being investigated along with effects of pertussis toxin on other aspects of insulin action.

2. Interaction of Calmodulin with Phosphodiesterase and other Proteins

We previously modified calmodulin with N-succinimidyl pyridyl dithiopropionate (SPDP) in order to link it covalently to calmodulin-binding proteins. To define the sites of modification, we have used N-succinimidyl propionate (^{3}H-NSP), a congener lacking the disulfide moiety. Data are consistent with the conclusion that Ca^{2+} binding to calmodulin exposes or enhances reactivity of two specific sites apparently located in a single CNBr fragment. When calmodulin was bound to melittin-Sepharose (a new affinity matrix prepared for purification of calmodulin and also useful for preparation of S-100 protein), five sites were labeled with ^{3}H-NSP. After elution, two more sites reacted with SPDP. Isolation of the thiol-containing peptide(s) should permit characterization of the domain that is shielded when calmodulin interacts with melittin.

The calmodulin-stimulated bovine brain phosphodiesterase (a homodimer of 59 kDa subunits) is activated by proteolysis with chymotrypsin, as we reported earlier.

Subsequent studies show that the activated phosphodiesterase, which cannot interact with calmodulin, behaves as a homodimer of ~ 45 kDa subunits. Chymotryptic cleavage in the presence of calmodulin plus Ca^{2+} likewise activates but yields a somewhat larger (47 kDa) peptide which, despite its ability to interact with calmodulin, is not further activated by it. After removal of Ca^{2+}, chymotrypsin converts the 47 to a 45 kDa species. The enzyme from ovine brain behaves in the same way (with slightly different sized peptides). All of the data are consistent with a phosphodiesterase model that distinguishes specific functional domains for catalysis, calmodulin binding, and inhibitory constraint.

High affinity antibodies to the purified phosphodiesterase react also with a second form of calmodulin-activated phosphodiesterase from bovine brain. The purified IgG was covalently linked to protein A-Sepharose yielding a matrix with which antigen can be rapidly separated and concentrated from crude fractions. This may permit final purification of the second enzyme for determination of the molecular basis of differences in the two forms.

Calcineurin, a calmodulin- and Ca^{2+}-binding protein, dephosphorylates several phosphoseryl- and phosphothreonyl-proteins as well as phosphotyrosine and p-nitrophenylphosphate (PNPP). Activity was compared using the latter substrate and a novel phosphotyrosyl derivative of glutamine synthetase (GSTP) prepared by Dr. T. Martensen, NHLBI. The enzyme was virtually completely dependent on Mn^{2+}, although Ni^{2+} (at considerably higher concentrations) could replace Mn^{2+}. Calmodulin increased activity 200-300% and Ca^{2+} had little effect. With PNPP, Lineweaver-Burk plots were nonlinear; at low substrate, calmodulin increased maximal velocity with little effect on K_m (25 mM). The phosphatase was very sensitive to inhibition by Zn^{2+} and orthovanadate but insensitive to fluoride. These characteristics as well as the relatively low K_m for GSTP are consistent with the possibility that calcineurin is a phosphotyrosyl-protein phosphatase. Although phosphatase activity was constant during assays without calmodulin, in its presence the high initial rate of catalysis declined rapidly, reaching a much lower, relatively constant rate after 4-5 min. This unusual deactivation occurred with both substrates and also when calcineurin was incubated with calmodulin prior to assay. Chymotryptic cleavage produced an activated species (twice as active as native calcineurin assayed with calmodulin) that was inhibited by calmodulin. These data suggest a modulatory rather than a strictly stimulatory role for calmodulin in regulation of calcineurin activity. Dimethylsulfoxide (30%) increased activity ~ 100% above that observed with maximal calmodulin activation, further indicating that, at least under the conditions employed, calmodulin does not support full expression of catalytic activity. In collaboration with Dr. M. L. Billingsley, NHLBI, it was found that enzymatic carboxymethylation of calcineurin and the phosphodiesterase decreased calmodulin activation but had little effect on basal activity. This type of selective modification may be useful in defining sites and mechanisms of calmodulin regulation.

3. Adenylate Cyclase

Hormone-sensitive adenylate cyclase systems include receptors for stimulatory and inhibitory ligands and stimulatory (G_s) and inhibitory (G_i) guanyl nucleotide-binding proteins through which agonist occupancy of receptors is translated into altered activity of the catalytic unit. G_s and G_i are heterotrimers consisting of a GTP-binding α-subunit ($G_{s\alpha}$ ~ 45 kDa and $G_{i\alpha}$ ~ 41 kDa) with identical β (35 kDa) and γ (6-10 kDa) subunits. It is believed that when GTP is bound the α-subunit is dissociated; $G_{s\alpha}$ can increase catalytic activity and $G_{i\alpha}$ may decrease it.

Hydrolysis of bound GTP leaves an inactive α-subunit with GDP bound. $G_{s\alpha}$ is known to possess GTPase activity and we have shown (see below) that $G_{i\alpha}$ is a GTPase. Agonist occupancy of receptors may promote replacement of GDP with GTP on G_s or G_i, thus accelerating GTP hydrolysis, i.e., increasing GTPase activity. Following our earlier work with cholera toxin, which activates adenylate cyclase by ADP-ribosylating $G_{s\alpha}$ and inhibiting its GTPase activity, we began last year to focus our attention on G_i and pertussis toxin, which abolishes receptor-mediated inhibition of cyclase by ADP-ribosylating $G_{i\alpha}$. Studies now include transducin, another GTP-binding regulatory protein. In retinal rod outer segments, the photon receptor rhodopsin acts through transducin to activate a cGMP phosphodiesterase. Transducin, like G_s and G_i, has a GTP-binding α-subunit ($T_\alpha \sim 39$ kDa) associated with β and γ subunits of 35 and $\sim$ 10 kDa. Other workers have shown structural similarities between both α and β subunits of transducin and G_i. With the goal of understanding at a molecular level the function of the adenylate cyclase system and the control of synthesis of its multiple components, an increasing fraction of our effort will be directed toward cloning the relevant genes. Because of the very small amounts of cyclase proteins present in cells, transducin, which can readily be purified in reasonable quantities, is being used for initial experiments, with the expectation that at least some of the results may be applied directly to work on G_i.

At the time this project was begun no information about the amino acid sequence of transducin was available. We found that the α and β subunits had blocked N-termini and could not be directly sequenced. Peptides from both were prepared by enzymatic cleavage and partial acid hydrolysis. One peptide from the α-subunit and two from the β as well as the first 26 amino acids of the γ-subunit have been sequenced, providing information from which suitable oligonucleotide probes are being prepared. Rabbit antisera to transducin, T_α, and $T_{\beta\gamma}$ have been screened for cross-reactivity with G_i subunits on Western blots. An antiserum to $T_{\beta\gamma}$ reacts with G_β but not G_γ. One monoclonal antibody (IgG_{2A}) specific for T_α inhibits the GTPase activity of T_α reconstituted with $T_{\beta\gamma}$ and rhodopsin (see below). Several other monoclonal antibodies to transducin are being characterized and screened for cross-reactivity with subunits of G_i.

We found last year that, on incubation of NG108-15 cells with pertussis toxin, opiate stimulation of adenylate cyclase decreased in parallel with opiate inhibition of GTPase. Measurements of ligand binding to opiate receptors suggested that, after ADP-ribosylation by pertussis toxin, G_i is effectively uncoupled from R_i, explaining the abolition of opiate effects on cyclase and GTPase. We have found that pertussis toxin also ADP-ribosylates the α-subunit of transducin (T_α) in photolyzed rod outer segment membranes with resultant inhibition of its GTPase activity. Effects of pertussis toxin were compared with those of choleragen which ADP-ribosylates T_α at a different site and inhibits GTPase activity. Using purified components reconstituted with phospholipids, it was found that, in the presence of $T_{\beta\gamma}$ and photolyzed rhodopsin, ADP-ribosylation of T_α by pertussis toxin was decreased by stable GTP analogues that promote dissociation of transducin subunits; i.e., the inactive associated holotransducin is apparently the preferred toxin substrate. Similar studies were carried out with the purified subunits of G_i which can interact functionally with rhodopsin to exhibit light-stimulated GTPase activity (see below). In the presence of photolyzed rhodopsin and $G_{\beta\gamma}$, effects of guanyl nucleotides indicated that, like T_α, $G_{i\alpha}$ was a better pertussis toxin substrate when it was associated with $G_{\beta\gamma}$ in the inactive G_i complex.

It has been reported that trypsin can cleave T_α to a 38 and then to a 32 kDa peptide. We find leucine at the N-terminus of both 38 and 32 kDa peptides; T_α is apparently blocked. Tryptic digestion of T_α that was [^{32}P]ADP-ribosylated with pertussis toxin yielded a labeled 38 kDa doublet followed by appearance of ^{32}P at the dye front. Others have reported that the toxin ADP-ribosylates an asparagine five amino acids from the C terminus. Therefore, it appears that removal of an N-terminal 1 kDa fragment produces the 38 kDa peptide followed by loss of a C-terminal 6 kDa segment to yield the 32 kDa peptide. The 38 kDa peptide from unmodified T_α, although it probably contains the pertussis toxin acceptor site, was a poor substrate perhaps because it does not interact effectively with $T_{\beta\gamma}$.

The separated subunits of transducin do not exhibit GTPase activity. As shown by Fung, the GTPase activity of T_α is evident after reconstitution with $T_{\beta\gamma}$ and photolyzed rhodopsin. Using subunits of G_i purified from rat liver, we found that $G_{\beta\gamma}$ could replace $T_{\beta\gamma}$ in this system. $G_{i\alpha}$ exhibited rhodopsin-stimulated GTPase activity, proportional to $G_{i\alpha}$ concentration, when reconstituted with $G_{\beta\gamma}$ or $T_{\beta\gamma}$. These studies demonstrate that the GTPase activity of G_i resides in $G_{i\alpha}$ and establish that $G_{i\alpha}$ and $G_{\beta\gamma}$ are functionally analogous to T_α and $T_{\beta\gamma}$, respectively. Rhodopsin stimulation of GTP hydrolysis by $G_{i\alpha}$ in the presence of $G_{\beta\gamma}$ may be compared with the receptor-mediated stimulation of GTPase by inhibitory ligands. The G_i-rhodopsin interaction could resemble the interaction of G_i with inhibitory receptors in the nature of the sites involved as well as in the manner by which it increases GTP hydrolysis by $G_{i\alpha}$. Some similarity or homology between rhodopsin and inhibitory receptors may be inferred.

4. Regulation of cAMP and cGMP Metabolism in Intact Cells

Bradykinin (BK) increases cAMP content and production of PGE_2 and PGI_2 by cultured human fibroblasts. Our earlier study of fibroblast BK receptors (the first characterization of BK receptors on human cells) showed that incubation with BK decreased receptor number without altering affinity. We recently found that dexamethasone and other glucocorticoids have a similar effect. Thus, they may reduce responsiveness to BK by decreasing receptor number as well as by inhibiting arachidonate release and prostaglandin synthesis. Following addition of BK to fibroblasts, PGD_2 was produced and degraded within 90 s. Immediately thereafter PGE_2, PGI_2, and cAMP content increased. When cellular phospholipids were labeled by incubation with [^{14}C]arachidonate and [^{3}H]myoinositol, it appeared that the initial effect of BK on PGD_2 was associated with hydrolysis of phosphatidylinositol and phosphatidylinositol-bis-phosphate by phospholipase C and release of arachidonate from diglyceride. Subequently, there was evidence for phospholipase A degradation of phosphatidylinositol correlated with production of PGE_2 and PGI_2. Whereas exogenous PGE_2 and PGI_2 elevated fibroblast cAMP content, PGD_2 did not but inhibited BK-induced increases in cAMP, PGE_2, and PGI_2. Thus, it seems that PGD_2 may play a role in modulating BK effects. BK-induced increases in PGE_2, PGI_2, and cAMP were enhanced after incubation of fibroblasts for 24 h with agents, including pertussis toxin, that increase cell cAMP content. These findings suggest another way in which BK responsiveness can be altered and may at least in part explain the enhanced sensitivity to BK that has been reported in animals given pertussis toxin.

In addition to effects on cAMP, BK causes a rapid transient increase in fibroblast cGMP content. Nitroprusside (NP), a drug that, like BK, causes vasodilation, also increases cGMP in many tissues. In fibroblasts, the transient rise in cGMP induced by NP was ten times that observed with BK, whereas the rise in cAMP was much smaller and there was no significant effect on PGI_2. From studies with inhibitors

of prostaglandin synthesis, it appeared that whereas effects of BK or BK plus NP on cAMP are mediated through PGI_2 or other products of arachidonate, effects on cGMP may be independent of or even inhibited by arachidonate metabolites. Responses to BK plus NP differed quantitatively and temporally from the sum of effects of BK and NP alone. Through interactions of this type, in vivo responses to drugs like NP may be influenced by BK or similar endogenous mediators. Effects of NP on segments of rat aorta or vena cava in vitro were compared with those of nitroglycerine, another vasodilator. Differences in the effects of the drugs on cGMP and PGI_2 production in the two tissues were consistent with and may contribute to clinically observed differences in their effects on arterial and venous circulations.

5. ADP-Ribosyltransferases

We have previously purified and characterized two NAD:arginine ADP-ribosyltransferases (termed A and B) from turkey erythrocyte cytosol. Transferase A can aggregate to an inactive, high molecular weight species that becomes activated upon dissociation promoted by chaotropic salts or histone. Since this enzyme appears to exist in membrane-bound as well as soluble forms, we investigated the effects of phospholipids on its activity. Lysophosphatidylcholines rather specifically increased activity (up to 500%) and their effectiveness was dependent on the nature of the fatty acid moiety. Other lysophospholipids and phospholipids, including phosphatidylcholine, did not activate. Certain detergents enhanced activity but not as effectively as lysolecithin. Those lysolecithins that were effective activators also stabilized the enzyme, consistent with other evidence that their effects result from interaction with the transferase rather than with substrates. Diverse stimuli increase lysolecithin production in cells and could perhaps thereby stimulate protein ADP-ribosylation.

Arginine residues critical for catalysis have been identified in a number of enzymes by reaction with arginine-selective reagents. We used glutamine synthetase (ovine brain), which contains an active site arginine reactive with such reagents, to determine whether this arginine might also be modified by transferase A. Incorporation of 0.9 mol of ADP-ribose/mol of synthetase was associated with 90% inhibition. The presence of substrate (MgATP), which protects against chemical modification of the critical arginine, decreased ADP-ribosylation and inactivation. Similar results were obtained with glutamine synthetase from chicken heart. It is appealing to speculate that the specificity of the transferase-catalyzed modification reflects an intracellular role for such enzymes.

A third NAD:arginine ADP-ribosyltransferase (transferase C) has now been identified in membranes from turkey erythrocytes. Although it catalyzes the same model reactions as transferases A and B, it is clearly different in physical and enzymatic properties as well as in subcellular localization. Another transferase localized to a highly purified nuclear fraction was found to be otherwise very similar to transferase A. The existence of this family of ADP-ribosyltransferases is consistent with multiple roles for ADP-ribosylation in cell function.

ADP-ribose-protein bonds have been categorized as labile or stable to neutral hydroxylamine. The labile bond was believed to be ADP-ribose-glutamine, and we showed that the stable bond corresponded to ADP-ribose-arginine. Investigation of the ADP-ribose-asparagine bond synthesized by pertussis toxin in transducin revealed that it represents a third class of bond that is more stable than ADP-ribose-arginine to hydroxylamine. The ADP-ribose linkage to $G_{i\alpha}$ was as stable as that in transducin, consistent with the probability that pertussis toxin modifies

an analogous asparagine in this protein. Similarly, stable ADP-ribose-protein bonds were found after incubation of membranes from human erythrocytes or NG108-15 cells with [^{32}P]NAD, suggesting the existence of ADP-ribosyl-(asparagine)protein transferases in addition to the previously described ADP-ribosyl-(arginine)protein transferases in animal cells.

6. Fatty Acid Metabolism in Cells from Patients with Lipid Abnormalities

These studies involved collaboration with investigators from NCI and other institutions. Cultured skin fibroblasts from patients with adrenoleukodystrophy contain greater than normal amounts of very long chain fatty acids but no defect in their transport or metabolism was demonstrable. Addition of oleic acid to the medium decreased the amount and synthesis of these fatty acids in cells from patients as well as normals, suggesting a possible mechanism for influencing tissue content of very long chain fatty acids. Patients with Sjögren-Larsson syndrome have abnormally low serum levels of fatty acids produced by desaturation of linolenic acid. In preliminary studies last year, the rate of desaturation of linoleate by cultured fibroblasts from two patients appeared somewhat less than that of control cells. However, further investigation with cells from four patients has established no significant defect in this activity. Samples of epidermal scales from nine patients with different types of hyperproliferative scaling disorders were analyzed for long chain hydrocarbons and very long chain fatty acids. A relatively high content of both was found in samples from patients with lamellar ichthyosis and congenital ichthyosiform erythroderma. Further investigation of such abnormalities may provide new information on normal pathways of fatty acid metabolism as well as understanding of the pathogenesis of these syndromes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00606-13 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of cAMP Content and Prostaglandin Production of Cultured Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous work showed that incubation of fibroblasts with bradykinin (BK) at 37°C markedly decreased the number of [3-H]BK-binding sites with little or no change in Kd for BK. The number of binding sites was also reduced by exposure (4-24 h) to dexamethasone (0.1-1 μM) or other corticosteroids with little or no change in Kd. Since corticosteroids are also known to inhibit release of arachidonate metabolites and production of prostaglandins, they can apparently reduce responsiveness to BK by receptor and post-receptor mechanisms.

Increases in fibroblast cAMP content and changes in prostaglandin formation influenced by culture conditions or perhaps BK itself also affected BK responsiveness. Following addition of BK, PGD-2 formation increased, then rapidly declined. Following the decline, there was a rapid rise in PGE-2 and PGI-2 production and cAMP accumulation. Whereas exogenous PGE-2 and PGI-2 increased cAMP content, exogenous PGD-2 inhibited effects of BK on prostaglandin formation and cAMP accumulation, suggesting a role for PGD-2 in modulating BK responsiveness. Using radiolabelling in conjunction with radioimmunoassay, we found that basal and BK-induced prostaglandin formation was derived primarily from slowly turning-over precursor pools and that BK stimulation caused hydrolysis of phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol.

In human fibroblasts, alterations in cGMP and cAMP accumulation and PGI-2 production in response to combinations of BK and nitroprusside (NP) differed quantitatively and temporally from the effects of BK and NP alone. In rat aortic and vena cava segments, different effects of NP and NG on cGMP content and PGI-2 production were consistent with differences in the effects of these two drugs on arterial and venous circulations.

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Project Description:

Objectives: To elucidate mechanisms whereby hormones and other effectors influence cyclic nucleotide metabolism and prostaglandin production in cultured cells and vascular smooth muscle; to specifically study the mechanism of bradykinin (BK) on prostaglandin formation and subsequent stimulation of cAMP and cGMP accumulation.

Methods Employed: Cultured fibroblasts were grown and maintained under standard conditions. Rat aorta and vena cava segments were prepared in Krebs-Ringer Tris buffer, pH 7.4, containing 3 mg/ml bovine serum albumin and 1 mg/ml glucose at 0-5°C and incubated at 37°C.

Cyclic nucleotides and prostaglandins were quantified by radioimmunoassay. Phospholipids, arachidonate metabolites, and prostaglandins were also analyzed by radiolabelling techniques.

For radiolabelling experiments, confluent fibroblasts were incubated with ^{14}C-arachidonate and ^{3}H-myoinositol in Eagle's minimal essential medium supplemented with 25 mM Hepes, pH 7.4, and 2 mM glutamine at 37°C in 5% CO_2 atmosphere for 3 or 18 h. Cells were washed twice with warm Hanks' medium (HBSS) and incubated at 37°C with 2 ml HBSS buffered with 25 mM Hepes, pH 7.4, with or without 0.15 μM BK, A-23187 (0.1 mg/ml), 10 μM isoproterenol, 2.8 μM PGE_2, 2.8 μM PGI_2, and/or 2.8 μM PGD_2. To stop the reaction, medium was then removed and 2 ml cold 5% trichloroacetic acid (TCA) was added to the culture dishes followed by freezing of the cells on the culture dishes. The incubation media were transferred to polypropylene tubes and assayed immediately for release of ^{14}C-dpm and ^{3}H-dpm as well as for the presence of specific arachidonate metabolites, as described below. For radioimmunoassay of PGE_2, PGF_2, and TXB_2, the media could be stored at -20°C for several days prior to assay without significant decrease in values. Both PGD_2 and 6-keto-PGF_1, however, were assayed immediately since immunoreactivity declined during storage. For analysis of cell proteins, cyclic nucleotides, and phospholipids, the cells were recovered from the dishes by thawing, scraping the dishes with a disposable plastic scraper, and rinsing with 2 ml HBSS. The cell suspension was then vortexed and used for assay of cell protein, cyclic nucleotide content, and for extraction of cellular lipids using the procedures outlined below.

Extraction of Cellular Lipids and Separation of Lipid Classes: Cell suspensions (2 ml in 2.5% TCA) were extracted with 5 ml chloroform, 10 ml methanol, and 0.5 ml concentrated HCl. The samples were subsequently separated into lipid classes by TLC on HPTLC silica gel plates using hexane:diethyl ether:acetic acid (83:16:1, v/v/v) as the mobile phase. Following development, the plates were dried at room temperature 15 min and the lipids visualized by exposure to iodine vapors for 2 min in a heated chamber to which iodine crystals were added. Lipids were identified by cochromatography with reference lipids and the radiolabel associated with each class determined by scraping the appropriate spots on the silica gel plates into scintillation vials and counting the silica gel in an LKB scintillation counter following addition of 10 ml Biofluor to the counting vials.

Extraction and Separation of Arachidonate Metabolites: The formation of arachidonate metabolites from ^{14}C-arachidonate-labeled fibroblasts was also

monitored by separation of the radioactive metabolites by TLC following extraction of the incubation medium. Medium was extracted with ethyl acetate:isopropanol:0.2 N HCl (3:3:1, v/v/v) in a ratio of 3 ml/ml of sample followed by vortexing twice for 15 s and addition of 2 ml ethyl acetate and 3 ml H_2O. Arachidonate metabolites were subsequently separated on silica gel GHL Uniplates, which were developed using the upper phase from an ethyl acetate:H_2O:acetic acid:iso-octane mixture (110:100:20:50, v/v/v/v) previously equilibrated 3 h prior to removal of the organic phase. After development, the TLC plates were dried for 30 min at room temperature and run a second time in the same solvent system. Arachidonate and metabolites were identified by cochromatography with appropriate reference compounds. The lipids were visualized by exposure of the TLC plates to iodine vapor and associated radioactivity was determined as described above.

Major Findings: We found earlier that interaction of BK with specific receptors on intact cultured human fibroblasts leads to activation of phospholipases A_2 and C and production of prostaglandins. Incubation of fibroblasts with glucocorticoids for several hours reduced these effects of BK and reduced the number of specific [^{3}H]BK-binding sites (up to 50%). K_d values for [^{3}H]BK binding were similar in control (3 nM) and steroid-treated (2.6 nM) cells. A 24-h incubation was necessary for maximal reduction. Steroids added to binding assays had no effect. Effects were observed with 1 nM dexamethasone and were maximal with 2 μM. Potencies of steroids in reducing [^{3}H]BK binding correlated well with their anti-inflammatory activities. Glucocorticoid-induced reduction in BK receptor number was unaffected by cycloheximide (5 μg/ml), which, however, by itself reduced receptor numbers. By decreasing receptor number, glucocorticoids may render target cells less sensitive to BK stimulation.

Conditions that alter prostaglandin production also alter BK effects on cAMP. When confluent fibroblasts were maintained for 14 days without replacement of growth medium, responsiveness to BK declined with no decline in the number of [^{3}H]BK-binding sites or affinity for BK. Responsiveness to BK was restored by addition of fresh growth medium.

Exposure of fibroblasts for 24 h to agents which increase cAMP content potentiated the BK-induced increase in PGI_2 and cAMP. After incubation of cells for 24 h with pertussis toxin (PT), which inactivates the inhibitory GTP-binding protein of adenylate cyclase, basal cAMP content was increased and the increase induced by subsaturating BK was enhanced. As the effect of exogenous prostaglandins on cAMP was unchanged, the increase in BK-stimulated cAMP formation did not apparently result from increased sensitivity to endogenous prostaglandins. Following incubation with PT, however, a 100-200% increase in BK-stimulated PGI_2 and PGE_2 production was observed at subsaturating but not at maximally effective concentrations of BK. The effect of PT on PGI_2 and PGE_2 production was in part mimicked by 8-Br cAMP. These studies are compatible with the hypothesis that increased BK sensitivity following prolonged PT treatment results from enhanced prostaglandin production in response to subsaturating concentrations of BK. The increase in prostaglandin formation may be secondary to activation of adenylate cyclase by PT, leading to increased cellular cAMP. These findings may in part account for the observation that BK sensitivity in experimental animals is enhanced by injection of pertussis toxin.

Prostaglandins produced in response to BK may also be involved in regulation of BK effects on cyclic nucleotide content and arachidonate metabolism. After exposure of fibroblasts to BK, PGD_2 measured by radioimmunoassay increased 10-fold within 60 s and declined to basal levels by 90 s. PGD_2 was unstable in fibroblast medium, and by 90 s both PGD_2 and its metabolites declined. Within the same period, BK induced a transient increase and decline in cGMP and TBX_2 content. Following the decline in PGD_2 levels, there was a rapid rise in the production of PGI_2 and PGE_2. BK increased PGI_2 several hundredfold and PGE_2 ~ 10-fold. Associated with the increase in PGI_2 and PGE_2 was a 30-fold elevation in cAMP content. Whereas exogenous PGI_2 and PGE_2 both increased cAMP levels, exogenous PGD_2 did not alter basal cAMP content. PGD_2, however, inhibited the effect of BK on cAMP by 40-60% and reduced BK-stimulated PGI_2 and PGE_2 production by 90%. These results support a role for PGD_2 in modulating the effects of BK on PGI_2 and PGE_2 metabolism and thus on cAMP accumulation.

Nitroprusside, like BK, causes vasodilation and hypotension and alters cAMP and cGMP metabolism. Addition of BK or NP to fibroblasts caused a rapid increase in cGMP which reached a maximum at 30 s and then fell; the rise with NP was ten times that with BK. With NP plus BK, the level at 30 s was lower than with NP alone. At later times, however, effects of BK and NP were slightly more than additive and maximal cGMP levels were reached at 90 s. BK increased cAMP through enhanced PGI_2 production. NP caused a small early increase in cAMP without a significant effect on PGI_2. Up to 50 s, effects on PGI_2 and cAMP were greater with BK alone than with BK plus NP; after 2 min, the opposite was found. Increases in cAMP or PGI_2 with BK or BK plus NP were blocked by indomethacin or 5,8,11,14-eicosatetraynoic acid. These agents did not alter cGMP in the presence of BK plus NP but enhanced NP-stimulated cGMP accumulation by 40-50%. Thus, effects of BK or BK plus NP on cAMP appear to be mediated through PGI_2 or other products of arachidonate, whereas effects on cGMP may be independent of or perhaps slightly inhibited by arachidonate derivatives. Cellular responses to BK plus NP differed quantitatively and temporally from the sum of effects of BK and NP alone. Through interactions of this type, *in vivo* responses to drugs like NP may be influenced by levels of BK or similar endogenous mediators.

Nitroprusside (NP), nitroglycerin (NG), and similar drugs appear to cause vasodilation in part through a common biochemical pathway involving an elevation in tissue cGMP content. Based on the clinical efficacy of these agents, however, it appears that the arterial and venous circulations respond differently to the drugs. To compare the biochemical effects of these agents on arterial and venous tissues, cGMP content of segments of rat aorta and vena cava was quantified following exposure of NP or NG *in vitro*. Since these agents may also act through the formation of vasodilatory prostaglandins, prostacyclin (PGI_2) production was also determined. NP was more effective in increasing the cGMP content of aorta than of vena cava and more potent than NG in increasing cGMP content in both tissues. Basal PGI_2 formation by aorta was greater than ten times that by vena cava. NG increased PGI_2 formation by aorta. NP had no significant effect. PGI_2 formation by vena cava was not affected by NP or NG. For aorta, more so than for vena cava, incubation conditions were critical in determining the relative effects of NG and NP on PGI_2 formation and cGMP content. After prolonged incubation (90 min), the stimulatory effect of NG on cGMP and PGI_2 was increased in aorta but not in vena cava. These differences in effects of NP and NG on cGMP and PGI_2 production in

aorta and vena cava are consistent with and may contribute to differences in effects of the drugs on arterial and venous circulations.

As mentioned above, maximal accumulation of PGD_2, TBX_2, and cGMP in response to BK occurred within 45 s; by 90 s, concentrations of these products had declined to basal values. On the other hand, accumulation of PGE_2, PGI_2, PGF_2, and cAMP in response to BK was maximal after 2 min of exposure to BK. In an attempt to gain some insight concerning the regulation of these events, studies were performed in fibroblasts labelled with ^{14}C-arachidonate (AA) and ^{3}H-myoinositol. After incubation for 18 h with ^{14}C-AA, BK-induced release of ^{14}C-AA from cell phospholipids was tightly coupled to prostaglandin synthesis in the sense that the ratio of released ^{14}C-prostaglandins to ^{14}C-AA was much higher in BK-treated cells than in control cells. After 2 min of exposure to BK, however, the specific activity (dpm/ng) of PGI_2 and PGE_2 was lower than that in control cells, suggesting that the bulk of the PGI_2 and PGE_2 released from BK-treated cells was synthesized from an unlabelled pool(s) of phopholipid. Based on requirements for exogenous Ca^{++} and the hydrolysis of distinct phosphoinositides, the time course of release of prostaglandins also correlated with activation of both phospholipase C and phospholipase A_2. The early effects of BK, i.e., on cGMP, PGD_2, and TBX_2, correlated with a decrease first in the amount of ^{14}C and ^{3}H in phosphatidylinositol-4, 5-bis phosphate (PIP_2), and then, within 1 min, in phosphatidylinositol (PI). Within 90 s, there was a transient increase and decline in ^{14}C-diglyceride, ^{14}C-phosphatidic acid, and an increase in release of ^{3}H-myoinositol triphosphate. Since these changes occurred prior to any increase in labelled lyso PI and since changes in PIP_2 and PI were not dependent on exogenous Ca^{++}, it is likely that some early events induced by BK involve hydrolysis of PIP_2 and PI by phospholipase C enzymes and release of AA from diglyceride via diglyceride lipase. Within 1-2 min of exposure to BK, there was a marked increase in ^{3}H-lyso PI and decrease in ^{14}C-PI, suggesting that some of the later (i.e., > 2 min) effects of BK on cAMP, PGI_2, and PGE_2, which were dependent on exogenous Ca^{++} and could be blocked by mepacrine, involved activation of phospholipase A_2.

Significance to Biomedical Research and the Program of the Institute: Bradykinin, a potent stimulus for prostaglandin production in many cell types, is thought to play an important role in maintenance of vascular tone and permeability and in inflammatory processes. Human foreskin fibroblasts, the first human cells in which BK receptors have been characterized, should prove useful for investigation of regulation of responsiveness to BK by receptor and post-receptor mechanisms as well as cellular mechanisms of action for vasoactive and hypotensive agents.

Fibroblasts also constitute a good model system to study the coordinate and independent regulation of the responsiveness of the cyclase system to BK, hormones, and other effectors.

Proposed Course: Continued study on the mechanism of action of BK with emphasis on BK activation of the arachidonic acid cascade and regulation of cyclic nucleotide metabolism.

Publications:

Roscher, A.A., Manganiello, V.C., Jelsema, C.L, and Moss, J.: Receptors for bradykinin in intact cultured human fibroblasts. Identification and characterization by direct binding study. J. Clin. Invest. 72: 626-635, 1983.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00614-07 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction of Calmodulin with Phosphodiesterase and other Binding Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Randall L. Kincaid, Ph.D.	Research Pharmacologist	CM, NHLBI
Others:	Sharon J. Geyer, Ph.D.	Guest Worker	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The sites of protein interaction on calmodulin (CaM) and the regulation of cyclic nucleotide phosphodiesterase (PDE) and calcineurin (CN) by CaM were investigated using chemical and enzymatic modification procedures and limited proteolysis. A calcium-dependent increase in the reaction between CaM and N-succinimidyl propionate (NSP) suggests exposure of two sites with increased reactivity located on CNBr fragment-2. Selective derivatization during and after immobilization on melittin-Sepharose (a new affinity matrix for purification of CaM) may permit isolation of a specific binding-protein domain on CaM. Proteolysis of PDE with chymotrypsin in the presence of EGTA yields a homodimer of 80-90k Da which cannot interact with CaM and is fully activated; in the presence of CaM, somewhat larger species are produced which are activated yet capable of interaction with CaM. A model is proposed which distinguishes specific functional domains for catalysis, CaM binding, and inhibitory control on PDE. High affinity antibodies react with two forms of PDE and its proteolytic fragments but show no reaction with CN or several other proteins. A method for covalent attachment of purified IgG to Protein-A Sepharose was developed providing an efficient procedure for analysis of PDE from different sources. The phosphatase activity of CN was compared using p-nitrophenyl phosphate (PNPP) and phosphotyrosyl glutamine synthetase (GSTP). The enzyme was dependent on Mn^{2+} for activity and was further stimulated 3- to 4-fold by CaM. While the Vmax was much higher for PNPP, the lower Km for GSTP (3 μM vs. 25 mM) was consistent with a role for CN as a protein phosphatase. Phosphatase time-dependence showed an unusual activation-deactivation phenomenon observed only with CaM. Chymotryptic cleavage of CN produced a highly activated species which was inhibited by the addition of CaM. These data suggest a modulatory, rather than strictly stimulatory, role for CaM in regulation of phosphatase activity. Enzymatic carboxymethylation of PDE or CN suppressed CaM-dependent activity while having little effect on basal reaction rate. This selective effect on activity may allow studies on the mechanism and site of CaM-dependent regulation.

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Project Description:

Objectives: To determine the relationship between calmodulin (CaM) interaction with its binding proteins and enzyme activation, especially as regards the calcium dependence of these two events. To isolate and characterize the physical domains of interaction of CaM and its binding proteins. To prepare antibodies against these proteins for investigating aspects of their *in vitro* and *in vivo* regulation.

Methods Employed: Homogeneous CaM, phosphodiesterase (PDE), and calcineurin (CN) were prepared by previously described methods. Dansyl-CaM and pyridyl dithio propionyl CaM were synthesized as in previous reports. Propionyl-CaM was prepared by incubation of N-succinimidyl propionate (NSP) with CaM (1-8 mg/ml) in 0.1 M sodium phosphate, pH 6.7, containing 0.1 M NaCl and 1 mM $MnCl_2$, $CaCl_2$, or EGTA as indicated; unlabeled NSP was generously provided by Dr. John Inman (NIAID). Reactions were terminated with trichloroacetic acid (final concentration of 3%). Precipitated CaM was solubilized in a minimal volume of 0.2 M Tris·HCl, pH 8.0, and radioactivity determined to calculate the degree of incorporation.

Cyanogen bromide (CNBr) fragments of CaM were prepared by incubation of CaM (5-20 mg/ml) with a 300- to 3000-fold excess of CNBr in 6 M urea containing 0.2 N HCl. After 1-2 h, 50 mM Tris, pH 8.0, containing 0.2 N NaOH was added and the mixture was chromatographed on Sephadex G-75, equilibrated in 50 mM NH_4HCO_3, pH 8.5; fractions were monitored for tyrosine fluorescence and radioactivity.

Proteolytic fragments of PDE were prepared as described in previous reports, and interaction with dansyl-CaM was measured using polarization of fluorescence as reported.

Antibodies were raised in rabbits (PDE) and in goat (CN). Rabbits were injected subcutaneously with ~ 100 µg of purified PDE at 10-day intervals. Preimmune and immune blood was collected via the ear vein. Sera were diluted with an equal volume of 80% glycerol and stored at -20°C. A goat was injected with 1 mg of purified CN at 10-day intervals and, after antibodies were present, plasmaphoresis was carried out. Plasma was diluted with an equal volume of 80% glycerol and stored at -20°C.

The titer and specificity of antibodies were estimated by nitrocellulose blotting methods. After SDS gel electrophoresis of antigen or crude sample, proteins were transferred electrophoretically from the gel to nitrocellulose paper (Western blot). The paper was blocked to reduce nonspecific binding, incubated with an appropriate dilution of antiserum or purified antibody (1:300-1:10,000) for 1-12 h, and then incubated with a detecting antibody specific for the species of the primary antibody covalently coupled to horseradish peroxidase; e.g., goat antirabbit peroxidase. Colored products appear after incubation with substrate.

Carboxymethylation of PDE and CN was carried out by incubation of these proteins with a 100-fold excess of purified protein carboxymethyl transferase (PCM) in 50 mM MES buffer, pH 6.3, containing 5-30 µM ^{3}H-S-adenosylmethionine. After 15 min, reactions were terminated by addition of an excess of the inhibitor, S-adenosylhomocysteine. Portions were precipitated with trichloroacetic acid for determination of radioactivity or assayed for enzyme activity at pH 6.5.

Major Findings: 1) Chemical Modification of CaM and Analysis of CNBr Fragments: We had previously modified CaM with succinimidyl pyridyl dithiopropionate (SPDP) in order to cross-link it to several of its binding proteins. To characterize the region(s) modified in CaM, we investigated the reaction of CaM with N-succinimidyl propionate (^{3}H-NSP), a congener lacking the reactive dithiopyridyl moiety of SPDP. The initial rate of modification at pH 6.7 was approximately twice as fast in the presence of Ca^{2+} as in its absence; later rates appeared similar. Total incorporation of propionate at different temperatures or NSP concentrations was always greater when Ca^{2+} was present (5-7 sites) than it was with EGTA (3-5 sites), consistent with the hypothesis that the Ca^{2+}-dependent conformation might expose or enhance the reactivity of two specific sites. Analysis of CNBr peptides of CaM was carried out after Ca^{2+}-dependent labelling with ^{3}H-NSP and indicated a specific increase in radioactivity for peptide CNBr-2, which contains three of the seven lysyl residues. This peptide was labelled, but to a much lesser degree, in the presence of EGTA, indicating that it was the reaction rate, and not the absolute accessibility of these sites, which was changed. To assess whether interaction with a binding protein might alter the availability of reactive sites, CaM was immobilized on melittin-Sepharose (see section #4) and incubated with ^{3}H-NSP. When the reaction was complete (i.e., when effluent radioactively plateaued), ^{3}H-CaM was eluted with EGTA and contained 4.5-5.5 mol ^{3}H-propionate/mol CaM. Further incubation with SPDP caused incorporation of 1.2-1.7 mol pyridyldithiopropionate (PDP)/mol CaM, suggesting that the immobilization of CaM may have protected 1-2 reactive sites at or near the interaction domain. Since this PDP/propionyl CaM would contain reactive sulfhydryl groups presumably only at the interaction domain, a convenient way to isolate and characterize this region may be to fragment the derivatized protein and chromatographically separate the sulfhydryl-containing regions. In addition, this derivative may be useful as a probe for interaction sites on other CaM-binding proteins.

2) Physical and Enzymatic Studies of CaM-activated Phosphodiesterase: In an extension of previous studies, the physical and enzymatic properties of bovine and ovine brain PDE were characterized before and after proteolysis. Bovine PDE (59 kDa) was cleaved, and activated by, trypsin (36 kDa), chymotrypsin (45 kDa), pronase (36 kDa), and papain (36, 45 kDa) in the presence of EGTA; the size of the final stable peptide is indicated in parentheses. The chymotryptic product, studied in detail, appeared to be a homodimer with $M_r \sim 80\text{-}90{,}000$, $S_{w,20}$ 4.55 S, Stokes radius of 3.9 nm, and a frictional ratio (1.34) virtually identical to that of the native enzyme. This species was not retained on CaM immobilized to Sepharose nor did it form a complex with a fluorescent congener, dansyl-CaM, indicating that it was no longer capable of interaction with CaM. As reported previously, when proteolyzed in the presence of Ca^{2+} and CaM, novel final peptides were observed for both bovine and ovine enzyme, consistent with exposure of a new proteolytic site on PDE resulting from CaM binding. Despite the ability of CaM to interact with these proteolytic species, they were fully activated in the presence of metal chelator. Since the chymotryptic peptides produced in the presence of CaM were slightly larger (47 kDa bovine, 42 kDa ovine) than those in its absence (45, 38 kDa, respectively) and could be cleaved to peptides of the latter sizes when EGTA was subsequently added, it suggests that a domain for CaM binding is vicinal to a region which imposes inhibitory constraint on PDE activity but that they are functionally independent.

Studies in collaboration with Dr. M. L. Billingsley, NHLBI, demonstrated substoichiometric carboxymethylation of bovine brain PDE by a purified carboxymethyl transferase from bovine brain. The inability to show stoichiometric modification presumably reflects the extreme lability of the methyl ester which spontaneously hydrolyzes at neutral pH. During methylation, CaM-dependent activity decreased with no change in basal PDE activity, suggesting that this modification may selectively affect interaction with CaM.

Antibodies against bovine and ovine PDE have been obtained in two rabbits. Using quantitative nitrocellulose blotting and electrophoretic transfer (Western blots), the titer and specificity of the antibodies were determined. The titer obtained after two injections of antigen (~ 5000) has not decreased for several months in both animals. Neither antiserum cross-reacts with CN, the catalytic subunit of CaM-dependent protein kinase, purified rod outer segment PDE, or with several unidentified CaM-binding proteins, although known proteolytic fragments of the PDE are recognized. An affinity-purified (Protein-A Sepharose) IgG fraction from rabbit sera showed only heavy and light chains of immunoglobulin on SDS gel electrophoresis. This preparation was as effective as serum in quantitative titrations while the serum fraction not retained on Protein-A Sepharose showed no reactivity; the half-maximal reaction occurred at an IgG concentration of 10^{-9} M, thus the interaction constant of the PDE-specific IgG was probably ~ 10^{-10} M. Quantitative chromatography on IgG covalently coupled to glutaraldehyde-activated Protein-A Sepharose (see section #4 below) indicated that 50-70 µg of PDE could bind per mg IgG, suggesting that a minimum of 6-10% of the IgG was specific for PDE, assuming stoichiometric association. Two forms of CaM-activated PDE from bovine brain both interacted with, and were eluted from, the IgG affinity column. Further study will determine the molecular basis for the previously noted differences in chromatographic and isoelectric behavior of the two PDEs.

3) Regulation of Phosphatase Activity of CN. The phosphotyrosyl protein phosphatase activity of CN was investigated in collaboration with Dr. Todd Martensen using denatured, monomeric ^{32}P-phosphotyrosyl glutamine synthetase (GSTP), which has a single phosphotyrosyl site per subunit. Phosphatase activity was virtually dependent on Mn^{2+}, with very low activity in the presence of Ca^{2+} or Ca^{2+}/CaM. The Mn^{2+}-dependent activity was stimulated ~ 3-fold by CaM and this was inhibited by CaM antagonists, W-7 and trifluoperazine. The phospho amino acids all inhibited activity at a concentration of 10-20 mM with phosphotyrosine somewhat more effective than phosphothreonine or phosphoserine. Mn^{2+}-dependent activity was constant with time in the absence, but not in the presence, of Ca^{2+} and CaM. In the presence of CaM, an initial phase of high activity lasting for 2-3 min was followed by a dramatic decrease in rate. This apparent "deactivation" was independent of amount of substrate hydrolyzed or enzyme concentration. It was not abolished by prior incubation of enzyme incubated for 5 min at 30°C, suggesting that it reflects a CaM-dependent regulatory property of the phosphatase. Analysis of substrate concentration dependence at 2 min and 12 min showed no difference in apparent K_m for GSTP (3 ± 1 µM), indicating that deactivation was not due to a large decrease in the affinity for substrate. Chymotrypsin activated CN concomitant with production of several smaller peptides (47, 45 kDa) of the A subunit (61 kDa). After proteolysis, activity in the presence of CaM was two to three times that of native enzyme assayed in the presence of CaM. Addition of CaM to assays of protease-treated CN depressed activity to the level observed with native enzyme in the presence of CaM, and it thus appears that CaM may play

a complex modulatory role, i.e., inhibitory as well as stimulatory, in regulation of phosphatase activity.

Calcineurin activity with p-nitrophenyl phosphate (PNPP), in studies carried out by Dr. Sharon Geyer, was similar to that with the phosphoprotein. V_{max} for PNPP (2-3 μmol/min/mg protein) was 200-300 times greater than that for GSTP. However, the K_m for PNPP was 10,000 times higher, consistent with a phosphoprotein phosphatase role for CN. Like chymotryptic cleavage, dimethylsulfoxide (20-30%) increased activity to twice that of the CaM-activated native enzyme. Thus, it appears that latent phosphatase activity, not observed in the presence of CaM, can be expressed under conditions that relieve inhibitory control (e.g., proteolysis of an inhibitory domain, solvent-induced structural deformation).

Bovine brain protein carboxymethyl transferase (PCM) modified CN to the extent of ~ 2 mol methyl ester/mol CN; this apparently represents the first substrate for which stoichiometric incorporation has been documented. The catalytic A subunit (61 kDa) of CN was the site of modification with little, if any, methyl ester present in the smaller (18 kDa) B subunit based on SDS gel electrophoresis at pH 2.4. Hydrolysis of CN methyl ester, while quite rapid at pH 7.5 ($t_{1/2}$ ~ 3 min), was slower at pH 7.0 and 6.3 ($t_{1/2}$ ~ 18 and 30 min, respectively). Since CN activity at pH 6.5 was 20-25% of maximal activity (pH 8) with no discernible change in regulation by metals or CaM, this pH was used to assay control and methylated CN; assay for 3 min insured that minimal loss of methyl ester occurred. Carboxymethylation of CN (1-2 mol CH_3/mol CN) dramatically reduced (70-80%) CaM-dependent activity with little effect on Mn^{2+}-supported "basal" activity, suggesting a selective interference with CaM activation.

Goat antibodies against CN appear to be specific for the A subunit based on Western blot analysis and have little, if any, cross-reactivity with PDE, the catalytic subunit of cAMP-dependent protein kinase, and several other proteins. These antibodies appear to have much lower affinities than those against PDE, requiring overnight incubation to bind to antigen. The amount of CN antibody is low as well (titers of 300-700). Analysis of Protein-A Sepharose-purified IgG suggests that < 1% of IgG is specific for CN. Purification of "mono-specific" IgGs using a CN-affinity column has been attempted. However, thus far, IgG eluted from such columns appears to be inactive or, at best, not enriched in CN-specific forms.

4) Development of Affinity Chromatography Methods. Two new procedures have been developed. The first greatly facilitates purification of CaM and S-100 protein. The second permits analysis of antigen-antibody interactions in crude samples as well as affinity purification of antigen.

Melittin, a 3 kDa bee venom peptide known to form high affinity complexes with CaM, inhibited CaM stimulation of purified PDE (K_i ~ 10-30 nM). The peptide was covalently linked to CNBr-activated Sepharose (0.6-10 mg melittin per ml gel). This affinity gel selectively retained CaM from crude tissue fractions in a Ca^{2+}-dependent fashion; bound protein was eluted with EGTA. The apparent capacity of this gel for CaM was directly proportional to substituent concentration, indicating no steric hindrance at higher levels of substitution nor changes in recovery or nonspecific interactions. The functional capacity of the affinity gel was 15-18% of the theoretical, indicating that, on the average, one-fifth of the

immobilized melittin was available for interaction. The high capacity (10-12 mg CaM/ml gel) and virtually quantitative recoveries (90-100%) permit rapid, specific concentration and purification of CaM from crude extracts. Another Ca^{2+}-binding protein in brain, S-100, was also retained albeit with lower affinity; prior or subsequent adsorption of this protein to organomercurial agarose removed it from the CaM fraction. Thus, an improved procedure (yield 200-250 mg/kg tissue) was developed for purification of CaM from bovine and ovine brain which yields a homogenous protein totally devoid of nucleotide absorbance. It involves extraction of whole brain in 6 M urea, followed by batchwise absorption to anion-exchange gel. After elution, the S-100 is removed by passage over a bed of organomercurial agarose, and unretained CaM is adsorbed to and eluted from melittin-Sepharose. If desired, the eluate from organomercurial agarose can be adsorbed to, and eluted from, melittin-Sepharose to yield pure S-100 protein (~ 200 mg/kg tissue).

Anti-PDE IgG was coupled to glutaraldehyde-activated Protein-A Sepahrose after incubation with various concentrations of glutaraldehyde and subsequent washing; a one-hour incubation with 1-2% glutaraldehyde appeared optimal. The covalent nature of IgG coupling was verified by extensive washing of the matrix with pH 3.5 buffers and buffers containing 6 M urea. Neither the capacity of Protein-A Sepharose for IgG nor that of bound IgG for PDE was substantially altered by this procedure, indicating preservation of their functional properties. After adsorption of PDE, the antigen-antibody complex could be dissociated with a variety of eluants including 2 M $MgCl_2$ (which permits recovery of enzyme activity), 6 M urea, etc. No antibody was detected in any of these eluates, and nonspecific adsorption appeared to be minimal based on SDS gel electrophoresis. In the case of high affinity antibodies, IgG immobilization provides a rapid method for concentration of antigenic material from crude fractions and, after elution, immunologic analysis of adsorbed material. This is not usually possible with crude supernatant or tissue extracts, since the amount of antigen is too low to permit analysis by SDS gel electrophoresis/Western blot immunoanalysis. Since this approach is suitable for all IgG of the appropriate subclass, and the adsorbent (at least for anti-PDE IgG) can be reused without loss of capacity, it appears to be generally promising for immunologic analysis.

Significance to Biomedical Research and the Program of the Institute: The effects of many biochemical regulators, such as hormones, on mammalian cells are mediated by altering the rates of synthesis and/or degradation of cyclic nucleotides and by the phosphorylation/dephosphorylation of certain cellular proteins. Investigation of the properties of the enzymes regulating these procedures is important for the understanding of normal and pathologic cellular activity and may permit design of rational therapeutic approaches. The well-established significance of CaM in control of diverse cellular functions makes the elucidation of its mechanism of action important for the understanding of calcium-regulated processes. The development of new biochemical methods (e.g., novel affinity chromatography approaches) may provide useful tools for investigation of basic biochemical and enzymologic process.

Proposed Course: To establish the relationship between the Ca^{2+} dependence of CaM interaction with its binding proteins and enzyme activation using fluorescence and cross-linking approaches as well as direct Ca^{2+}-binding studies. To isolate and characterize the binding protein domain(s) on CaM after specifically

modified thiol-containing regions are prepared; similar approaches will be used for CaM-binding regions on the binding proteins. To use immunologic methods to survey tissues and cultured cells for the content and subcellular localization of PDE and CN.

Publications:

Vaughan, M., and Kincaid, R.L.: Mechanism of action of cyclic nucleotide phosphodiesterase by calmodulin: Utilization of novel biologically active derivatives of calmodulin to probe mechanisms of Ca^{2+}-dependent interaction and enzyme activation. Sixth US-USSR Symposium on Myocardial Metabolism, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00622-07 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Cyclic Nucleotide Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others:	Robert E. West, Ph.D.	Staff Fellow	CM, NHLBI
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COOPERATING UNITS (if any)

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SECTION

Molecular Mechanisms

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.8	1.8	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1) Adenylate cyclase activity is under the control of inhibitory and stimulatory agonists which act through specific surface receptors. The inhibitory receptors are coupled to the catalytic unit of cyclase through a guanine nucleotide-binding protein termed Gi. Specific binding of [3-H]guanyl-5'-yl imidodiphosphate (Gpp(NH)p) was observed to membranes from cultured NG108-15 neuroblastoma x glioma hybrid cells; since inhibitory agonists induced release of Gpp(NH)p, it appeared that the binding was to Gi. Pertussis toxin, which catalyzes the ADP-ribosylation of Gi, reduced the inhibitory agonist-dependent release. To determine the mechanism for this effect, the binding of [3-H]Gpp(NH)p to ADP-ribosylated and unmodified transducin, a retinal protein similar to Gi, was examined. ADP-ribosylated transducin bound significantly less Gpp(NH)p than did the unmodified protein. Thus, the failure of agonist to release Gpp(NH)p from membranes from toxin-treated cells results from the inability of guanine nucleotide to bind. 2) NAD:arginine ADP-ribosyltransferases which catalyze reactions similar to bacterial toxins, were previously identified in the cytosolic fraction of turkey erythrocytes. One such enzyme, termed transferase A, was activated by membrane components, such as lysophospholipids, as well as by nonionic and zwitterionic detergents; lysophospholipids are generated in response to activation of phospholipase A-2. This transferase modifies the arginine residues of proteins. In the case of ovine brain glutamine synthetase, ADP-ribosylation of one arginine resulted in loss of enzymatic activity; the ADP-ribosyltransferase thus recognizes specific arginine residues and may therefore have a role in regulating enzymatic function. In addition to the cytosolic ADP-ribosyltransferases A and B, two different transferases, termed C and A', were localized to the membrane and nuclear fractions, respectively. These transferases possess different physical, kinetic, and regulatory properties. The existence of a family of ADP-ribosyltransferases is consistent with a multifaceted role for mono-ADP-ribosylation in the cell.

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Project Description:

Objectives: To study the regulation of cyclic nucleotide metabolism. The hormone-sensitive adenylate cyclase system is responsible for the synthesis of cyclic AMP. Studies on the activation of adenylate cyclase by the bacterial toxins choleragen and pertussis toxin have defined the roles of two guanine nucleotide-dependent regulatory proteins (termed G_s and G_i, respectively) responsible for the control of catalytic unit activity; these toxins exert their effects on cells by catalyzing the transfer of the ADP-ribose moiety of NAD to critical amino acid residues on these regulatory proteins. Pertussis toxin appears to enhance adenylate cyclase activity by blocking the action of inhibitory agonists. These agents, acting through specific cell surface receptors, depress catalytic unit activity; the inhibitory receptor is coupled to the catalytic unit through the guanine nucleotide-binding protein, G_i, a pertussis toxin substrate. ADP-ribosylation of G_i by toxin blocks the coupling of inhibitory receptor to catalytic unit and thereby promotes the unopposed action of the stimulatory arm of the adenylate cyclase system. To further clarify the mechanism of cyclase activation by pertussis toxin, the effect of toxin-catalyzed ADP-ribosylation on the specific binding of guanine nucleotides to G_i was examined in cultured cells; the investigations were then continued with a purified protein from retinal rod outer segment, transducin, which appears to be homologous to G_i in structure and function and serves as a pertussis toxin substrate. Although the importance of toxin-catalyzed ADP-ribosylation was apparent from these and prior studies, the roles and regulation of endogenous ADP-ribosyltransferases have not been defined. In the present studies, we have examined factors that activate an endogenous NAD:arginine ADP-ribosyltransferase; this enzyme, like choleragen and *E. coli* heat-labile enterotoxin, catalyzes the transfer of ADP-ribose to the amino acid arginine, other low molecular weight guanidino compounds, and proteins. We examined whether the NAD:arginine transferase could specifically inactivate enzymes possessing arginine residues critical for activity. These studies have also resulted in the identification of a membrane-bound ADP-ribosyltransferase, different from the cytosolic enzyme described earlier. Thus, there appears to be a family of NAD:arginine ADP-ribosyltransferases.

Methods Employed: 1) Assays: NAD glycohydrolase and ADP-ribosyltransferase assays were performed by modifications of methods developed in this laboratory. Glutamine synthetase was assayed by published procedures.

2) Enzyme Purification: NAD:arginine ADP-ribosyltransferase "A" was purified from turkey erythrocyte supernatant by methods developed in the laboratory. Glutamine synthetase from chicken heart was purified by published procedures.

3) Pertussis Toxin: The toxin was purified by published procedures.

Major Findings: 1) Effect of Pertussis Toxin-catalyzed ADP-ribosylation of the Inhibitory Guanine Nucleotide-binding Protein of Adenylate Cyclase on its Interaction with Guanyl-5'-yl Imidodiphosphate: The hormone-sensitive adenylate cyclase system consists of stimulatory and inhibitory receptors linked through different guanine nucleotide-binding proteins to a catalytic unit responsible for the conversion of ATP to cAMP; the guanine nucleotide-binding proteins are known as G_s and G_i for those participating in stimulation and inhibition, respectively. G_s and G_i are heterotrimers consisting of α, β, and γ subunits. The α subunits

bind guanine nucleotide and appear to be in the active conformation when containing bound GTP or a GTP analogue. Hydrolysis of the bound GTP by a GTPase activity intrinsic to the α subunit results in the formation of protein-bound GDP; experimental evidence is consistent with the hypothesis that G_i and G_s containing GDP or its analogues are inactive. Agonists apparently promote the conversion of the inactive to the active state by facilitating the release of GDP and thus permitting the binding of GTP. To understand the mechanism of the release, guanyl nucleotide binding was examined in membranes prepared from cultured NG108-15 (neuroblastoma x glioma hybrid) cells; these cells possess inhibitory and stimulatory receptors and both types of guanyl nucleotide-binding proteins. [^{3}H]Gpp(NH)p was bound specifically to membranes from these cells; release was obtained with agonists that are inhibitors of adenylate cyclase (muscarinic cholinergic and α_2-adrenergic agents, opiates), consistent with the hypothesis that the [^{3}H]Gpp(NH)p was bound to the inhibitory guanyl nucleotide-binding component of the cyclase system. Release of [^{3}H]Gpp(NH)p was greater with two or three agonists than with one and was dependent on free guanyl nucleotide. ADP-ribosylation of G_i by pertussis toxin, which uncouples the inhibitory receptor from G_i, reduced the total binding of [^{3}H]Gpp(NH)p to membranes and the specific release of guanyl nucleotide in response to inhibitory agonists. Since pertussis toxin-catalyzed ADP-ribosylation of transducin, a protein structurally and functionally related to G_i, significantly decreased its binding of [^{3}H]Gpp(NH)p, it appears that the absence of agonist-stimulated release from membranes containing ADP-ribosylated G_i may be related to decreased guanyl nucleotide binding.

2) Regulation of ADP-ribosyltransferase Activity: Conditions have been defined that regulate the activity of the NAD:arginine mono-ADP-ribosyltransferases. An NAD:arginine ADP-ribosyltransferase from erythrocytes exists in an inactive aggregated form of high molecular weight that becomes activated upon dissociation. The conversion from the inactive to the active form was promoted by chaotropic salts or histones. The activity of the transferase thus appears to be sensitive to local environment and quaternary structure. Since the enzyme appears to exist in soluble and membrane-bound forms, the effect on transferase activity of membrane constituents such as phospholipids was investigated.

The activity of the erythrocyte NAD:arginine ADP-ribosyltransferase was enhanced ~ 6-fold by phospholipids; both animal (egg yolk) and plant (soybean) lysolecithins were highly effective as were lysophosphatidylcholines containing palmitoyl (C_{16}) > stearoyl (C_{18}) > C_{14} > C_{12} > C_{10} $\cong$ C_8 side chains; lysophosphatidylglycerol, lysophosphatidylserine, lysophosphatidylethanolamine, and lysophosphatidic acid did not increase transferase activity; glycerophosphatidylcholine and choline were inactive. It thus appeared that both the fatty acid and choline moieties are critical to the activation. No effect was observed with phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid.

Activation of the ADP-ribosyltransferase by lysolecithin was rapid and reversible. Lysolecithin also stabilized the enzyme against thermal denaturation. Those lysolecithins (C_{18}, C_{16}, C_{14}) that were effective activators of the enzyme also stabilized; C_{12} was clearly less active while C_{10} was inactive. Activation was not necessarily associated with stabilization, since 300 mM NaCl, which enhances enzyme activity, did not prevent thermal inactivation.

Lysolecithin increased the ADP-ribosylation of both low molecular weight guanidino compounds and model protein acceptors; the ability of lysolecithin to cause a stimulation of protein ADP-ribosylation was highly selective. Lysolecithin also stimulated NAD hydrolysis to the same extent as the ADP-ribosylation of agmatine. It was noted previously that the activity of the erythrocyte transferase was enhanced by chaotropic salts or histone. Lysolecithin was clearly not as effective as either of these agents in activating the transferase; lysolecithin did not further stimulate transferase assayed at maximally effective salt or histone concentrations.

Detergents were examined for their ability to increase ADP-ribosylation. The zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) and certain nonionic detergents (e.g., Triton X-100, Triton X-114, Tween 20, Triton X-305) enhanced enzymatic activity. As with lysolecithin, the detergents also stabilized the transferase against thermal denaturation. Maximal activation by CHAPS was less than that obtained with lysolecithin, histone, or NaCl. In the presence of CHAPS and lysolecithin, activity was only slight less than that obtained with NaCl or histone alone.

The structural requirements in the phospholipid and detergent in order to observe activation of the transferase may reflect those features necessary to form an effective interface for stabilization of the active conformation of the transferase. A direct effect of lysolecithin on the transferase is supported by the finding that, in the absence of protein and low molecular weight guanidino compounds, lysolecithin stimulated the transferase-catalyzed hydrolysis of NAD to ADP-ribose and nicotinamide. The observed increase in stability in the presence of lysolecithin also supports direct binding of the transferase to lysolecithin micelles.

The role of lysolecithin in the activation of transferase in vivo is unclear. Generation of lysophosphatidylcholine by the action of phospholipase A_2 may enhance the ADP-ribosylation of critical arginine residues in proteins. Since phospholipase A_2 activity is increased by hormones such as bradykinin, conceivably these agents could exert their effects on cells in part through an increase in ADP-ribosylation.

3) Effect of ADP-ribosylation on Enzymatic Activity. Animal cells contain endogenous enzymes that catalyze the mono-ADP-ribosylation of proteins and arginine, reactions analogous to those catalyzed by the bacterial toxins choleragen and E. coli heat-labile enterotoxins. The role of toxin-catalyzed ADP-ribosylation in the activation of adenylate cyclase was elucidated in studies from this and other laboratories. The function of the endogenous NAD:arginine mono-ADP-ribosyltransferases in the metabolism of animal cells has not been defined. Prior studies have noted that the transferases are selective in the use of proteins as ADP-ribose acceptors. We investigated whether the transferases might preferentially modify certain arginine residues.

A number of enzymes have critical arginine residues that participate in catalysis. These have been identified by the use of chemical reagents that specifically react with arginine. Glutamine synthetase from ovine brain, which contains an active site arginine that is reactive with such reagents, was used

to determine whether this arginine might also be preferentially modified by an ADP-ribosyltransferase.

Glutamine synthetase from ovine brain was inactivated by incubation with NAD and NAD:arginine ADP-ribosyltransferase. ADP-ribose and nicotinamide, products of the enzyme-catalyzed hydrolysis of NAD by the transferase, were inactive. NADP, which is utilized much less efficiently by the transferase, was not an effective substitute for NAD. Agmatine, an alternate ADP-ribose acceptor in the transferase-catalyzed reaction, prevented the inactivation of glutamine synthetase. Addition of MgATP, previously shown to block chemical inactivation of synthetase by arginine-specific reagents, reduced the rate of inactivation of synthetase by transferase and NAD; protection of the enzyme by this substrate is consistent with the arginine residue being located at or affected by occupancy of the catalytic site. The transferase-catalyzed inactivation of glutamine synthetase was associated with the transfer of [^{32}P]ADP-ribose from [^{32}P]NAD to the enzyme. Maximal inhibition of glutamine synthetase activity of 90%, reached after incubation of this enzyme with transferase for ~ 1 h, was associated with transfer of ~ 0.89 ± 0.07 mol ADP-ribose per mol glutamine synthetase. The ratio of mol of [^{32}P]ADP-ribose per mol of glutamine synthetase to (%) inactivation was ~ 0.99.

The selective modification and inactivation by ADP-ribosylation of glutamine synthetase was not limited to the enzyme from ovine brain. The glutamine synthetase purified from chicken heart was also inactivated by the transferase in an NAD-dependent reaction. ADP-ribose and nicotinamide could not replace NAD. Inhibition of the chicken heart enzyme by transferase was maximal by 1 h and was dependent on the amount of transferase present. As noted with the ovine enzyme, both agmatine and MgATP protected the synthetase from inactivation and also decreased the extent of ADP-ribosylation. In the presence of [^{32}P]NAD, 0.60 ± 0.03 mol ADP-ribose was transferred per mol glutamine synthetase, resulting in a 95% inactivation of the enzyme. The ratio of mol of [^{32}P]ADP-ribose per mol of glutamine synthetase to (%) inactivation was 0.63.

It is evident from the data that the NAD:arginine ADP-ribosyltransferase may be used as a reagent to catalyze the covalent modification of arginine residues in both pure protein and tissue homogenates. The ADP-ribosyl(arginine) protein bond is relatively stable in acid and at physiological pH. In tissue homogenates, enzyme(s) responsible for degradation of the ADP-ribosyl(arginine) protein bond, if they exist, are relatively inactive under standard assay conditions and thus unlikely to present a threat to the stability of the ribosyl(arginine) protein linkage. Reversal of transferase-like reactions requires low pH (5.5-6.0) and high concentrations of nicotinamide; it is thus unlikely to proceed under physiological conditions. Since phosphodiesterases that catalyze the degradation of the ADP moiety are common, to tag arginine residues in crude extracts, it would be preferable to use NAD labeled in the nicotinamide ribose; phosphodiesterase and phosphatase action on ADP-ribose(arginine) protein would result in the formation of ribose(arginine) protein as a radiolabeled end product. Since the transferase-catalyzed ADP-ribosylation is specific for certain arginine residues, this reaction may not be utilized with proteins in their native conformation; denaturation of the protein substrate may enhance the availability of other arginine groups to the transferase and thus increase the extent of ADP-ribosylation.

In previous studies, it was shown that ADP-ribosylation was affected by nucleotides such as GTP or ATP which, depending on the protein, either increased, decreased, or had no effect on the rate of modification. These experiments did not demonstrate an effect of ADP-ribosylation on function. From the present study, it is clear that MgATP blocks the inactivation of glutamine synthetase. Although it was uncertain from the previous studies whether the modification of the proteins had any selectivity other than the presence of a "readily accessible" arginine, the present investigation demonstrates that the transferase-catalyzed reaction can be specific for certain arginine residues. Of the 25 arginine .esidues in ovine brain glutamine synthetase, the erythrocyte transferase selectively modified that residue critical for enzymatic activity. It is clear from model studies on the transferase-catalyzed ADP-ribosylation of arginine and other low molecular weight guanidino compounds that the environment of the guanidino is a critical determinant of its ability to serve as an ADP-ribose acceptor. The presence of negatively charged residues in the vicinity of the guanidino moiety decreased its reactivity in the transferase-catalyzed reaction; agmatine and arginine methyl ester were more effective substrates than were arginine or guanidinopropionate. In addition, the reactivity of an arginine in a protein is in part determined by the nucleophilicity of the guanidino group and thus its ability to displace nicotinamide from NAD^+. The secondary and tertiary structures of glutamine synthetase serve as determinants of the pK of the guanidino moiety. A decrease in pK would enhance the reactivity of the guanidino group with phenylglyoxal and at the catalytic site of the transferase. In the case of the transferase-catalyzed reaction as opposed to chemical modification, however, the picture is complicated by the fact that the substrate for the transferase is another protein; in order for the critical arginine to be modified, the catalytic site of glutamine synthetase must be accessible to the active site on the transferase. It is thus appealing to speculate that the specificity reflects an in vivo significance for this reaction and a function for the NAD:arginine ADP-ribosyltransferase.

4) Identification of NAD:Arginine ADP-ribosyltransferases in the Membrane and Cytosolic Fractions of Turkey Erythrocytes: Previous studies have disclosed the occurrence in turkey erythrocytes of two cytosolic enzymes termed transferases A and B which, like choleragen and E. coli heat-labile enterotoxin, are NAD:arginine mono(ADP-ribosyl)transferases. Recently, a third such enzyme, transferase C, was identified in membrane fractions from turkey erythrocytes. The enzyme was extracted from a thoroughly washed particulate fraction with 0.5 M NaCl and purified 50,000-fold by sequential chromatography on phenyl-Sepharose, carboxymethyl-cellulose, concanavalin A-Sepharose, NAD-agarose, and DNA-agarose. In addition to differences in cellular localization and chromatographic behavior, transferase C was distinguished from transferases A and B by: (1) different chromatographic properties than the cytosolic enzymes; (2) a K_{av} on gel permeation columns intermediate to those of transferases A and B, indicating a molecular weight of approximately 30,000 Da; and (3) slight (< 70%) salt or histone stimulation of activity in contrast to a marked (10- to 20-fold) activation of transferase A by either and a salt inhibition of transferase B activity. In addition, another transferase was localized to highly purified nuclear fractions of the turkey erythrocyte. This activity, termed A', is otherwise similar to the cytosolic transferase A in terms of apparent molecular weight and salt or histone stimulation of its activity. These studies are consistent with the hypothesis

that there exists a family of ADP-ribosyltransferases having different intracellular localizations as well as different physical, kinetic, and regulatory properties and support a multifaceted role for mono(ADP-ribosylation) within the cell.

Significance to Biomedical Research and the Program of the Institute: The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by extracellular agents such as hormones and toxins. The lung, in particular, is exposed through the tracheobronchial tree to a variety of bacterial and toxic agents. To be effective, some of these agents must interact with a cellular receptor, and a substantial number of these agents exert their effects by altering the steady-state levels and localizations of cyclic nucleotides within the cell. A number of pharmacological agents have been in use which override aberrant physiological control to the benefit of the patient; these agents have been designed to interact with specific cellular receptors. By using cultured cells, model systems and purified preparations, it may be possible to simplify and define the factors critical to cyclic nucleotide metabolism. These models can then be used to understand the controls which operate in the more complex pulmonary and cardiovascular systems.

Proposed Course: (a) To study the regulation of G_i and transducin by toxin-catalyzed ADP-ribosylation using purified and membrane components; (b) to isolate and characterize the ADP-ribosyltransferases from animal cells; (c) to examine the role for ADP-ribosyltransferases isolated from animal cells in the regulation of enzymatic activity.

Publications:

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Moss, J., Watkins, P.A., and Yost, D.A.: Characterization of NAD:arginine ADP-ribosyltransferases in animal tissues. In Proceedings of 13th International Princess Takamatsu Cancer Research Fund Symposium, Tokyo, Japan, November 16-18, 1982. Japan Sci. Soc. Press, Tokyo/VNU Science Press, Utrecht, 1983, pp. 103-109.

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Moss, J., Osborne, J.C., Jr., and Stanley, S.J.: Activation of an erythrocyte NAD:arginine ADP-ribosyltransferase by lysolecithin and nonionic and zwitterionic detergents. Biochemistry 23: 1353-1357, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00625-06 CM

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
ADP-Ribosylation of Transducin by Pertussis Toxin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul A. Watkins, M.D., Ph.D.	Medical Staff Fellow	CM, NHLBI
Others:	Joel Moss, M.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI
	Yasunori Kanaho, Ph.D.	Visiting Fellow	CM, NHLBI

COOPERATING UNITS *(if any)*
Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Charlottesville, VA (Dr. Erik L. Hewlett).

LAB/BRANCH
Laboratory of Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Incubation of photolyzed bovine rod outer segment (ROS) membranes with pertussis toxin led to an inhibition of GTPase activity in an NAD-dependent reaction; the decrease in GTPase activity paralleled an increase in [32-P]ADP-ribosylation of a 39k Da protein which is the α-subunit of transducin (Tα). ADP-ribosylation of Tα occurred in both photolyzed and dark ROS membranes. Neither ATP nor Gpp(NH)p was required for ADP-ribosylation or inhibition of GTPase. Incubation of ROS with choleragen or pertussis toxin prevented subsequent [32-P]ADP-ribosylation by the same but not the other toxin; i.e., the two toxins label different sites on Tα. Tα, which is [32-P]ADP-ribosylated by pertussis toxin in purified transducin, was also labeled by the toxin after separation from Tβγ (36k Da and ~ 10k Da); neither component of Tβγ was a pertussis toxin substrate. Labeling of Tα was enhanced by Tβγ (maximally at a molar ratio of ~ 1:1).

Limited proteolysis by trypsin of Tα in the presence of Gpp(NH)p produced sequentially proteins of 38 and 32k Da. The amino terminus of both 38 and 32k Da proteins was leucine; that of Tα was apparently blocked. The 32k Da peptide was not a pertussis toxin substrate. Poor labeling of the 38k Da protein was poor and was not enhanced by Tβγ. Trypsin treatment of [32-P]ADP-ribosyl-Tα produced a labeled 37-38k Da doublet followed by appearance of 32-P at the dye front. Without rhodopsin, labeling of Tα (in the presence of Tβγ) was unaffected by Gpp(NH)p, GTPγS, GTP, GDP, or GDPβS but was increased by ATP. With photolyzed rhodopsin and Tβγ, Gpp(NH)p and GTPβS decreased labeling. Pertussis toxin ADP-ribosylates the inhibitory guanine nucleotide-binding subunit of adenylate cyclase (Gi) but not the stimulatory subunit (Gs); choleragen ADP-ribosylated Gs but not Gi. Since both toxins ADP-ribosylate Tα, it appears that this protein may possess characteristics of both Gs and Gi.

Project Description:

Objectives: Hormonal control of adenylate cyclase is mediated by receptors for stimulatory and inhibitory ligands that act through regulatory proteins, termed G_s and G_i, respectively. Since G_s and G_i are present in minute amounts in animal tissues, investigation of their properties has been limited. However, transducin, a protein that is structurally and functionally similar to G_s and G_i, is present in reasonable quantities in retinal rod outer segments (ROS) and is easily isolated. Transducin, G_s, and G_i are heterotrimers, each consisting of subunits designated α (39, 45, and 41k Da, respectively), β (36, 35, and 35k Da, respectively), and γ (5-10k Da). Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of $G_{s\alpha}$, resulting in inhibition of its GTPase activity which is normally increased by stimulatory hormones. ADP-ribosylation by choleragen of the α-subunit of transducin (T_α) in ROS, resulting in decreased GTPase activity, was recently reported. Pertussis toxin (islet-activating protein) ADP-ribosylates $G_{i\alpha}$, thereby preventing hormonal inhibition of adenylate cyclase and stimulation of GTPase activity of $G_{i\alpha}$. The objective of this project was to determine 1) whether T_α was a substrate for pertussis toxin-catalyzed ADP-ribosylation, 2) the effect of toxin on GTPase activity of transducin, 3) the requirements for labeling of T_α by the toxin.

Methods Employed: ROS, transducin, and rhodopsin were purified from bovine retinas by published procedures. T_α was separated from $T_{\beta\gamma}$ by chromatography on Blue-Sepharose. Pertussis toxin-catalyzed ADP-ribosylation was determined by incubation at 30°C with [^{32}P]NAD, followed by electrophoresis and radioautography. GTPase activity was measured by release of $^{32}P_i$ from [α-^{32}P]GTP. Limited proteolysis of T_α was carried out by incubation at 30°C with trypsin in the presence of Gpp(NH)p. N-terminal amino acids were identified as dansyl derivatives.

Major Findings: Incubation of photolyzed ROS membranes with NAD and pertussis toxin and/or choleragen decreased GTPase activity. The effects of the toxins were not additive. Inhibition of GTPase by choleragen reached a maximum by 2 h, whereas inhibition by pertussis toxin was slower and was still increasing at 4 h.

The effect of pertussis toxin on GTPase, like that of choleragen, was dependent on NAD, consistent with the hypothesis that it resulted from the ADP-ribosyltransferase activity of the toxin. Both pertussis toxin and choleragen catalyzed the [^{32}P]ADP-ribosylation of the 39k Da protein; work in other laboratories established that this protein is the α-subunit of transducin, T_α.

When ROS membranes were incubated with choleragen, 1 mM NAD, and 5 µM Gpp(NH)p overnight to ensure maximal ADP-ribosylation of T_α, addition of pertussis toxin and [^{32}P]NAD for 4 h resulted in [^{32}P]ADP-ribosylation of this protein. In contrast, virtually no [^{32}P]ADP-ribosylation was observed when either no toxin or choleragen was present during the 4-h incubation. A similar experiment in which the overnight incubation was with pertussis toxin revealed that addition of choleragen, but not fresh pertussis toxin, resulted in [^{32}P]ADP-ribosylation of T_α during the subsequent 4-h incubation. These results are consistent with the conclusion that pertussis toxin and choleragen catalyze the ADP-ribosylation of different sites on T_α.

Choleragen-catalyzed [^{32}[]ADP-ribosylation of T_α in ROS was dependent on Gpp(NH)p; ATP had no effect on the extent of ADP-ribosylation whether or not Gpp(NH)p was present. In contrast, [^{32}P]ADP-ribosylation of T_α in ROS by pertussis toxin was significant in the absence of added nucleotides, and labeling was only slightly increased when Gpp(NH)p (with or without ATP) was present. ATP alone decreased pertussis toxin-catalyzed [^{32}P]ADP-ribosylation.

In photolyzed ROS membranes, inhibition of GTPase by choleragen required Gpp(NH)p but was not significantly affected by ATP, whether or not Gpp(NH)p was present. With pertussis toxin, neither inhibition of GTPase activity nor ADP-ribosylation of T_α required Gpp(NH)p. In the presence of Gpp(NH)p, ATP had little effect on pertussis toxin-catalyzed ADP-ribosylation of T_α or inhibition of GTPase; in the absence of Gpp(NH)p, both were decreased by ATP.

Choleragen is reported to ADP-ribosylate poorly and nonspecifically all three subunits of purified transducin. Pertussis toxin-catalyzed ADP-ribosylation was specific for T_α and proceeded equally well in ROS membranes and in purified transducin. T_α, purified from transducin, was [^{32}P]ADP-ribosylated by pertussis toxin in the presence of ATP and Gpp(NH)p. $T_{\beta\gamma}$, which was not a pertussis toxin substrate, increased toxin-catalyzed [^{32}P]ADP-ribosylation of T_α in a concentration-dependent manner. At a molar ratio of $T_{\beta\gamma}$ to T_α of ~ 1.0, labeling of T_α was maximal and was ~ 5-fold greater than in the absence of $T_{\beta\gamma}$.

Limited proteolysis of T_α by trypsin in the presence of Gpp(NH)p results in the sequential formation of 38 and 32k Da proteins. The N-terminal amino acid of both the 38 and 32k Da proteins was leucine, whereas the N-terminus of T_α could not be identified and was presumed to be blocked. Compared to T_α, the 38k Da protein was a poor substrate for pertussis toxin; the 32k Da protein was not labeled. $T_{\beta\gamma}$, which enhanced [^{32}P]ADP-ribosylation of T_α, had no effect on labeling of the 38 and 32k Da proteins. When T_α was [^{32}P]ADP-ribosylated by pertussis toxin before trypsin treatment, significant label was found in a 37-38k Da doublet; no [^{32}P]ADP-ribosylated 32k Da protein was found.

In the absence of exogenous nucleotides, T_α was a poor substrate for pertussis toxin unless $T_{\beta\gamma}$ was present. Gpp(NH)p had no apparent effect on labeling of T_α (with or without $T_{\beta\gamma}$) in the absence of rhodopsin but caused marked inhibition when photolyzed rhodopsin was present. In contrast, ATP enhanced [^{32}P]ADP-ribosylation of T_α whether or not $T_{\beta\gamma}$ was present. Even with ATP, however, Gpp(NH)p inhibition was apparent in the presence of photolyzed rhodopsin. The stimulatory effect of ATP was detectable at concentrations as low as 5 μM.

Several other guanine nucleotides, e.g., GTP, GDP, and GDPβS, had little effect on [^{32}P]ADP-ribosylation of T_α (with or without $T_{\beta\gamma}$) in the presence or absence of photolyzed rhodopsin. GTPγS, which like Gpp(NH)p is a nonhydrolyzable analog of GTP, had effects similar to those of Gpp(NH)p.

In dark ROS, compared to photolyzed ROS, ADP-ribosylation of T_α by choleragen was minimal whether or not Gpp(NH)p was present. Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation was, however, similar, as were the effects of ATP and Gpp(NH)p, in dark and bleached ROS membranes. ADP-ribosylation by pertussis toxin of T_α in purified transducin proceeded identically under normal room lighting and in total darkness; similar results were obtained if purified T_α in the presence

of $T_{\beta\gamma}$ was the substrate rather than transducin. The inhibition of labeling by Gpp(NH)p in the presence of photolyzed rhodopsin was not observed with dark rhodopsin. The ability of $T_{\beta\gamma}$ to promote a labeling of T_{α} was likewise diminished in the presence of photolyzed rhodopsin and Gpp(NH)p.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the role of the guanyl nucleotide-binding regulatory proteins, G_s and G_i, in hormonal control of adenylate cyclase has been hampered by 1) the low levels of these protein animal tissues, 2) difficulty of purification, and 3) instability after purification. Transducin, the homolog of G_s and G_i found in retinal ROS, is not subject to these limitations. The effects of agents, such as choleragen and pertussis toxin, which are known to modulate adenylate cyclase activity by modifying G_s or G_i can be defined using transducin and the information obtained thereby applied to understanding regulation of the adenylate cyclase system which is central to cellular metabolism.

Proposed Course: 1) Define further the factors that influence choleragen and pertussis toxin-catalyzed ADP-ribosylation of purified T_{α}; 2) relate to the adenylate cyclase system pertinent observations on transducin.

Publications: Watkins, P.A., Moss, J., Burns, D.L., Hewlett, E.L., and Vaughan, M.: Inhibition of bovine rod outer segment GTPase by Bordetella pertussis toxin. J. Biol. Chem. 259: 1378-1381, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00627-06 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

GTP-Binding Proteins and Adenylate Cyclase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Su-Chen Tsai, Ph.D.	Research Chemist	CM, NHLBI
Others:	Yasunori Kanaho, Ph.D.	Visiting Fellow	CM, NHLBI
	Joel Moss, M.D., Ph.D	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.7	1.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adenylate cyclase systems consist of inhibitory and stimulatory receptors linked to a catalytic unit through inhibitory and stimulatory proteins, Gi and Gs, respectively. Similarly, the retinal photon receptor rhodopsin is linked with a phosphodiesterase through transducin (T), which is similar to Gs and Gi. Each has a guanyl nucleotide-binding α subunit (45 kDa Gs, 41 kDa Gi, and 39 kDa T) and β,γ subunit (35 and 10kDa). (1) The α and $\beta\gamma$ subunits of Gi, Gs, and T were purified. (2) To demonstrate the functional similarity between Gi and T, subunits of Gi were tested for their ability to replace the corresponding T subunits in the rhodopsin-stimulated GTPase reaction. GTPase activity was observed when the α subunit of Gi or T was combined with $\beta\gamma$ subunits of either Gi or T and rhodopsin. These data demonstrate (a) that Giα possesses intrinsic GTPase activity and (b) that photolyzed rhodopsin can mimic an inhibitory receptor-agonist complex in its ability to interact with Gi. (3) The purified α subunits of Gi and T are substrates for the pertussis toxin-catalyzed ADP-ribosylation reaction. ADP-ribosylation of Giα by pertussis toxin was much more effective in the presence of G$\beta\gamma$. Photolyzed rhodopsin mimicking agonist-inhibitory receptor complex inhibited the reaction. ADP-ribosylation of Giα with G$\beta\gamma$ and photolyzed rhodopsin was enhanced by GDP and GDPβS but not by Gpp(NH)p, Gpp(CH-2)p, or GTPγS; dark rhodopsin had only minimal effects on ADP-ribosylation. Since dark rhodopsin and guanosine diphosphate analogues favor the associated inactive Giα-G$\beta\gamma$ species, whereas photolyzed rhodopsin and nucleoside triphosphate analogues promote dissociation of Giα G$\beta\gamma$ to release the active forms, Giα and G$\beta\gamma$, these studies support the hypothesis that the preferred substrate for pertussis toxin is the inactive species, Giα G$\beta\gamma$.

Project Description:

Objectives: The hormone-sensitive adenylate cyclase system is critical to the regulation of cellular processes by hormones, toxin, and drugs. The system consists of stimulatory and inhibitory receptors linked through different guanyl nucleotide-binding regulatory proteins to a catalytic unit. It appears to be analogous to the retinal rod outer segment rhodopsin-transducin-cGMP phosphodiesterase complex, where rhodopsin is the photon receptor and transducin the guanyl nucleotide-binding coupling protein. The goals of this project are to define the mechanism of receptor-coupling protein interaction.

Methods Employed: Guanosine Triphosphate Hydrolysis: GTPase activity of transducin and G_i was determined by established procedures.

Major Findings: (1) Isolation of the Guanyl Nucleotide-Binding Proteins of the Adenylate Cyclase System. The adenylate cyclase system consists of inhibitory and stimulatory receptors linked to a catalytic unit through inhibitory and stimulatory GTP-binding proteins, G_i and G_s, respectively. Each has a GTP-binding α subunit (45 kDa in G_s and 41 kDa in G_i) and a 35 kDa β subunit, apparently identical in G_i and G_s, which is associated with 6-10 kDa γ subunit. The light-activated cGMP phosphodiesterase of retinal rod outer segments is analogous in several ways to the cyclase system. The photon receptor rhodopsin is linked with the phosphodiesterase through transducin, which consists of a GTP-binding α subunit (39 kDa) and $\beta\gamma$ subunits (35 and 10 kDa). To initiate the studies on the adenylate cyclase system, the guanyl nucleotide-binding proteins were isolated from rabbit liver membranes.

$G_{s\alpha}$ and $G_{i\alpha}$ were purified as described by Sternweis et al. Membrane proteins were solubilized in cholate and activated by addition of Al^{3+}, Mg^{2+}, and F^-. The supernatant from a 100,000 x g centrifugation was purified further by DEAE-Sephadex ion exchange chromatography, Ultrogel AcA 34 gel filtration, heptylamine Sepharose affinity chromatography, DEAE-Sephadex and hydroxylapatite chromatography. $G_{i\alpha}$, separated from G_s and $G_{\beta\gamma}$ during affinity chromatography on heptylamine Sepharose and further purified on hydroxylapatite, appeared to be > 90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. $G_{\beta\gamma}$ was separated from G_s and G_i during the second DEAE-Sephadex chromatography and was further purified on hydroxylapatite. The purity of $G_{\beta\gamma}$, estimated from Coomassie blue stained-sodium dodecyl sulfate-polyacrylamide gel, was > 90%.

(2) Effect of Rhodopsin on the GTPase Activity of Transducin and G_i: Manning and Gilman (J. Biol. Chem. 258: 7059-7063, 1983) showed that the α and β subunits of G_i are very similar in amino acid composition and protease digestion pattern to the corresponding subunits of transducin. The functional similarity between G_i and transducin was assessed by testing the ability of $G_{s\alpha}$ and $G_{\beta\gamma}$ to replace corresponding T subunits in the rhodopsin-stimulated GTPase reaction.

As noted by Fung (J. Biol. Chem. 258: 10495-10502, 1983), GTPase activity was observed when the isolated subunits of purified transducin, T_α and $T_{\beta\gamma}$, were combined in the presence of the light-activated rhodopsin. $G_{i\alpha}$ exhibited rhodopsin-stimulated GTPase activity when reconstituted with $G_{\beta\gamma}$ or $T_{\beta\gamma}$. $G_{\beta\gamma}$ replaced $T_{\beta\gamma}$ in reconstituting rhodopsin-stimulated GTPase activity of T_α. Dark rhodopsin possessed considerably less activity than photolyzed rhodopsin, with either $T_\alpha T_{\beta\gamma}$

or $G_{i\alpha}G_{\beta\gamma}$. These findings indicate that the subunits of G_i are functionally equivalent to those of transducin in the rhodopsin-stimulated GTPase reaction. Furthermore, it appears that the interaction of the G_i with rhodopsin is similar to the interaction of G_i with inhibitory receptor. Dark rhodopsin appears to be analogous to the inactive, unoccupied inhibitory receptor, whereas photolyzed rhodopsin mimicked the active agonist-receptor complex.

(3) Effect of Guanyl Nucleotides and Rhodopsin on ADP-Ribosylation by Pertussis Toxin of the Inhibitory Guanyl Nucleotide-Binding Component of Adenylate Cyclase. The $G_{i\alpha}$ subunit of the inhibitory guanyl nucleotide-binding component of adenylate cyclase was purified as described. ADP-ribosylation of $G_{i\alpha}$ by pertussis toxin was enhanced > 5-fold by $G_{\beta\gamma}$. ATP, previously shown to increase the ADP-ribosylation of $G_{i\alpha}$ in membrane preparations, enhanced ADP-ribosylation of $G_{i\alpha}$ in the absence more than in the presence of $G_{\beta\gamma}$. Labeling of $G_{i\alpha}$ in the presence of $G_{\beta\gamma}$ was increased slightly by guanyl nucleotides and to a lesser extent by ATP; GTP, GDP, and GDPβS were somewhat more effective than Gpp(NH)p, Gpp(CH_2)p, and GTPγS. Since prior studies on the GTPase activity of $G_{i\alpha}$ demonstrated that rhodopsin can substitute for the inhibitory hormone receptor, the effects of photolyzed and dark rhodopsin on the ADP-ribosylation of $G_iG_{\beta\gamma}$ by pertussis toxin were examined; photolyzed rhodopsin presumably mimicked the action of the agonist-receptor complex while nonphotolyzed rhodopsin served as the inactive, unliganded receptor. Pertussis toxin-catalyzed ADP-ribosylation of $G_{i\alpha}$ in the presence of $G_{\beta\gamma}$ was decreased by photolyzed but not dark rhodopsin. With photolyzed rhodopsin, GDP, GDPβS, and GTP significantly enhanced ADP-ribosylation; under these conditions, GTP was converted by the GTPase to GDP.

Stable, hydrolysis-resistant, GTP analogues, such as Gpp(NH)p, Gpp(CH_2)p, and GTPγS, when present with photolyzed rhodopsin had only small effects on ADP-ribosylation; GTP analogues promote the dissociation of G_i to the active forms, $G_{i\alpha}$ and $G_{\beta\gamma}$. Since GDP analogues promote formation of the inactive $G_{i\alpha}G_{\beta\gamma}$ complex, it appears that the preferred substrate for pertussis toxin is the associated form of G_i.

Significance to Biomedical Research and the Program of the Institute: The hormone-sensitive adenylate cyclase system is critical to the regulation of the cardiopulmonary system. Various inhibitory or stimulatory agonists affect cells by controlling the rate of cAMP generation; drugs currently in clinical use are directed at modifying the adenylate cyclase activity. Understanding the molecular basis for the interaction of these agents with adenylate cyclase-linked receptors and subsequently of the active receptor complexes with the coupling proteins and catalytic unit of cyclase may further the development and design of therapeutic agents.

Proposed Course: (1) Define the factors involved in the regulation of G_i and transducin activity, both with the purified proteins and in membrane preparations. (2) Determine the effects of agonist-receptor complexes or photolyzed rhodopsin on G_i and transducin activity.

Publications: Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E.L., Moss, J., and Vaughan, M.: Rhodopsin-enhanced GTPase activity of the inhibitory GTP-binding protein of adenylate cyclase. J. Biol. Chem. 259: 7378-7381, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00630-05 CM

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Metabolism of Fatty Acids in Fibroblasts from Patients with Lipid Abnormalities

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Joel Avigan, Ph.D. Research Chemist CM, NHLBI

COOPERATING UNITS *(if any)*
Department of Pediatrics, Medical College of Virginia (Dr. W. B. Rizzo); Department of Pediatrics, University of Umeå, Sweden (Dr. O. Hernell and co-workers); Dermatology Branch, NCI (Dr. G. L. Peck).

LAB/BRANCH
Laboratory of Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Skin fibroblasts from patients with adrenoleukodystrophy (ALD) have elevated hexacosanoic acid in their complex lipids. The concentration thereof is substantially reduced on adding 20-50 μM oleic acid to the medium. Oleic acid also reduces the C-26 fatty acid content in normal fibroblasts. Oleic acid was shown to inhibit synthesis of very long chain fatty acids (VLFA) from labeled acetate. The effect may indicate a mechanism for in vivo regulation of VLFA content in tissues and it could possibly be useful in treatment of patients with ALD.

2. An expanded series of fibroblast lines from 4 patients with Sjögren-Larsson Syndrome (SLS), a neurological disorder in which there are abnormally low levels of serum linolenic and arachidonic acids, was studied for Δ5- and Δ6-fatty acid desaturase activities. There was no significant difference between the activities in patient and control cells. It was shown in normal fibroblasts that the relative distribution of labeled arachidonic acid in various complex lipids is the same for the exogenous arachidonate taken up from medium and for that produced endogenously by desaturation and elongation of linoleic acid.

3. Samples of epidermal scales from 9 patients with several forms of hyperproliferative scaling disorders were analyzed for VLFA and long chain hydrocarbons. A relatively high content of both classes of compounds was found in some of the samples of epidermal scales.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives: 1) To investigate transport, metabolism and accumulation of very long chain fatty acids (VLFA) in normal skin fibroblasta and those from patients with adrenoleukodystrophy (ALD). 2) To investigate linoleic acid desaturation in human skin fibroblasts including the ones grown from patients with Sjögren-Larsson Syndrome. 3) To study fatty acid and hydrocarbon content and composition of skin scales of patients with various forms of ichthyosis.

Methods Employed: Conventional methods were used for growing human skin fibroblasts in culture. Isolation and quantification of long and very long chain fatty acid homologues were carried out by GC or by HPLC. In studies of linoleic acid desaturation, labeled linoleate was incubated with cell cultures and the resulting polyunsaturated fatty acid products were fractionated on argentated thin-layer plates. The hydrocarbon and fatty acid content and composition in ichthyosis patient skin scales was determined by GC fractionation.

Major Findings: 1) The previously reported reduction in VLFA content following addition of physiological concentrations of oleic acid to fibroblast cultures was further investigated. Both normal and ALD cells were affected. The effect of oleic acid was concentration-dependent with a maximal response at 20-50 μM. When ALD fibroblasts were incubated in lipid-free medium, which eliminated contribution from an exogenous source, oleate diminished concentration of C26:0 and C24:0 by 64% and 31%, respectively. The content of unsaturated VLFA (C22:1, C24:1) was unchanged. The effect of oleate on fibroblast content of C26:0, but not C24:0, was decreased, but not abolished, in lipid-containing medium. Synthesis of C26:0 from labeled acetate in normal and ALD fibroblasts was inhibited 20-50% by oleate. This effect was specific and not merely due to dilution of label by an increased pool of 2-carbon fragments derived from oxidation of oleate. These findings suggest that fatty acids (e.g., oleic acid) may have a physiological role in modulating cellular VLFA synthesis and content.

2) Following the preliminary experiments reported last year, we have further studied Δ^5- and Δ^6-fatty acid desaturase activity in fibroblasts derived from 4 patients with Sjögren-Larsson Syndrome (SLS), a condition associated with deficiency in γ-linolenic and arachidonic acids, and from a number of normal controls. Time course of desaturation of [1-^{14}C]linoleate was established. Assays of desaturase activity indicated the presence of Δ^5- and Δ^6-desaturases in SLS-derived cells at activity levels similar to that found in control fibroblasts. We have concluded that the reported abnormalities in the unsaturated fatty acid composition of sera of SLS patients do not seem to be due to a radical enzymatic defect. In normal cells that had been incubated with medium containing both labeled arachidonic and linoleic acids, the relative distribution in various complex lipids present in cells of the exogenous arachidonate and of that produced endogenously from linoleate was the same, indicating the metabolic identity of the product derived from both sources.

3) Sample scales collected from 9 patients with several types of hyperproliferative scaling disorders were analyzed for hydrocarbons and fatty acids. A high content of long chain alkanes (1-2% of dry weight) was found in samples from patients with lamellar ichthyosis and congenital ichthyosiform erythroderma. The largest alkane fraction was in the range of 25 and 28 carbon length. In the

fatty acid fraction, the proportion of VLFA was substantially higher in samples from the two conditions mentioned above than from that of psoriasis and epidermal hyperkeratosis. A precise interpretation of these results may require work with additional patient material.

Significance to Biomedical Research and the Program of the Institute: 1) Abnormal accumulation of various fatty acids is associated with certain congenital disorders, such ALD, characterized by accumulation of fatty acids greater than C-22 in patients' tissues. Factors causing reduction in concentration of these components may lead to useful therapy.

2) Polyunsaturated fatty acids are essential for cellular function, but the role of their endogenous production from linoleic acid in satisfying this requirement in cells of healthy and diseased individuals is not known.

3) Patients with certain conditions of abnormal lipid metabolism frequently show ichthyosis. Exploration of abnormalities in lipid composition and metabolism in patients with scaling diseases could produce information on factors associated with these conditions as well as on normal pathways of fatty acid metabolism in human tissues.

Proposed Course: 1) Studies of the effect of experimentally induced elevation of serum-free oleic acid on serum VLFA content in ALD patients are being considered.

2) The effect of senescence of human fibroblasts on Δ^5- and Δ^6-fatty acid desaturase activities is being investigated.

3) Fatty acid analysis of scales from additional patients with several types of ichthyosis will be carried out.

Publications:

Rizzo, W.B., Avigan, J., Chemke, J., and Schulman, J.D.: Adrenoleukodystrophy: Very long-chain fatty acid metabolism in fibroblasts. Neurology 34: 163-169, 1984.

Avigan, J., Askanas, V., and Engel, W.K.: Muscle carnitine deficiency: Fatty acid metabolism in cultured muscle and fibroblasts. Neurology 33: 1021 1026, 1983.

Avigan, J., Campbell, F., Yost, D.A., Hernell, O., Holmgren, G., and Jagell, S.F.: Sjögren-Larsson syndrome: Δ^5- and Δ^6-fatty acid desaturases in skin fibroblasts. Neurology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00634-04 CM

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of cGMP-Stimulated Cyclic Nucleotide Phosphodiesterase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hideo Wada, M.D., Ph.D.	Visiting Fellow	CM, NHLBI
Others:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*
Molecular Disease Branch, NHLBI (J. C. Osborne, Jr., Ph.D.)

LAB/BRANCH
Laboratory of Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.8	1.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In purifying the cGMP-stimulated cyclic nucleotide phosphodiesterase from calf liver, two "low Km" cAMP phosphodiesterases were separated from the cGMP-stimulated phosphodiesterase and from each other. One "low Km" form hydrolyzed cAMP with a Km of ~ 0.5 μM and was very sensitive to inhibition by cGMP and cilostamide; the other form hydrolyzed cAMP with a Km of ~ 2 μM and was sensitive to inhibition by Ro 20-1724. These two forms may be analogous to two "low Km" forms in rat adipose tissue and 3T3-L1 adipocytes. During differentiation of 3T3-L1 adipocytes, particulate low Km cAMP phosphodiesterase activity appears (or increases markedly) which can be activated by lipolytic hormones and insulin and is sensitive to inhibition by cGMP and cilostamide. Soluble cAMP phosphodiesterase activity in these cells is not apaprently hormone-sensitive and is inhibited by Ro 20-1724 and insensitive to inhibition by cilostamide and cGMP.

With the purified cGMP-stimulated phosphodiesterase, the competitive inhibitors papaverine, dipyridamole, and IBMX stimulated hydrolysis of 0.5 μM cAMP, whereas cilostamide did not. In addition, IBMX was a more effective inhibitor than theophylline. The competitive inhibitors which stimulated hydrolysis at low substrate concentrations mimicked substrate and brought about allosteric transitions that increased catalytic activity. Cilostamide inhibits, perhaps at catalytic sites, without apparently inducing the allosteric transitions. From studies with a series of xanthine analogs, derivatives with propyl substitutions at positions 1 and 3 of the purine nucleus are more effective inhibitors than those with methyl group substitutions (i.e., theophylline, 8-Cl theophylline and caffeine).

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Project Description:

Objectives: To characterize the physical and enzymatic properties and regulation of the cGMP-stimulated cyclic nucleotide phosphodiesterase (EC 3.4.1.17); to define effects of phosphodiesterase inhibitors on the purified enzyme and two other "low K_m" cAMP phosphodiesterases, with the possibility of identifying specific inhibitors that may be useful for investigating activity of individual phosphodiesterases in intact cells.

Methods Employed: Preparation of the Enzyme. Procedures for purification of the cGMP-stimulated phosphodiesterase from calf liver supernatant and verification of purity were described last year. Two "low K_m" cAMP phosphodiesterases which, unlike the cGMP-stimulated enzyme, did not bind to cAMP-agarose were separated by chromatography on AcA 34.

Major Findings: One form (C) of low K_m cAMP phosphodiesterase hydrolyzed cAMP with an apparent K_m of ~ 0.5 μM and was very sensitive to inhibition by cGMP. Lineweaver-Burk plots of cAMP hydrolysis by a second form (B) were nonlinear, with an apparent low K_m component of ~ 2 μM. This form was rather insensitive to inhibition by cGMP. With both B and C, hydrolysis of cAMP relative to cGMP was much greater at low (~ 1 μM) than at high (~ 100 μM) substrate concentrations. Maximal velocities for cAMP and cGMP were similar. From sedimentation equilibrium, the apparent weight-average molecular weight of B was estimated as 174,000 and that of C as 85,000. Another fraction (A) of cAMP phosphodiesterase eluted at the void volume of the AcA 34 column. On the basis of the relative affinities for cAMP and cGMP and inhibition by cGMP, A is most likely an aggregated form of B. No apparent interconversion of A, B, or C was observed on high-performance liquid chromatography. B and C differed in sensitivity to phosphodiesterase inhibitors as well as in other characteristics. The order of potency for inhibition of B was Ro 20-1724 (IC_{50}, 2.2 μM) > papaverine > isobutylmethylxanthine (IBMX) > cilostamide > theophylline > cGMP. The order for C was cilostamide (IC_{50}, 0.03 μM) > cGMP (IC_{50}, 0.75 μM) > papaverine > IBMX > theophylline > Ro 20-1724. These two forms may be analogous to two low K_m forms in rat and 3T3-L1 adipocytes, one sensitive to cilostamide and cGMP and capable of being activated by insulin and lipolytic hormones, the other apparently hormone-insensitive but sensitive to inhibition by Ro 20-1724. The use of specific inhibitors may facilitate understanding of the role of specific phosphodiesterases in the regulation of intracellular cAMP content.

We reported last year that three competitive inhibitors, papaverine, dipyridamole, and isobutylmethylxanthine (IBMX), can mimic substrate and effect allosteric transitions that increase catalytic activity of the cGMP-stimulated phosphodiesterase, whereas another, cilostamide, apparently cannot. Differences in the actions of these inhibitors presumably reflect differences in the molecular requirements for effective interaction at catalytic and allosteric sites on phosphodiesterase, i.e. differences in the structure of these sites. Since theophylline (1,3 dimethylxanthine) was much less potent an inhibitor than IBMX, it seemed that the isobutyl side chain which could interact at a hydrophobic site on the phosphodiesterase was important in the action of IBMX. A number of xanthine analogs substituted in the 1, 3, and 7 or 8 positions of the purine ring were supplied by Searle. Dixon analysis of kinetic data indicated that all were competitive inhibitors capable of increasing enzyme activity at low (0.5-2.5 μM)

cAMP concentrations. IBMX and compound 2627 (1,3 dipropylxanthine) were much more potent inhibitors than theophylline, 8-chloro theophylline, or caffeine (1,3,7-trimethylxanthine), suggesting that hydrophobic interactions may play an important role in the interaction of these inhibitors with the phosphodiesterase. Analogs further modified by substitutions at position 7 were usually less effective inhibitors than the parent compound, e.g., caffeine < theophylline; 5192 (1,3 dipropyl, 7 methylxanthine) < 2627 (1,3 dipropylxanthine). We have begun to analyze the kinetics of cAMP hydrolysis in the absence or presence of competitive inhibitors according to a simple interaction model for allosteric enzymes. With this approach, it may be possible to probe interactions of substrate and inhibitors at high or low affinity sites on the enzyme.

Significance to Biomedical Research and the Program of the Institute: Many biochemical effectors, such as hormones and drugs, exert their effects on target cells by altering metabolism of cyclic nucleotides. Understanding the regulatory properties of the phosphodiesterases involved in the degradation of the cyclic nucleotides is important for understanding mechanisms that regulate physiological and pathological processes in mammalian cells.

Proposed Course: Continued characterization of the enzyme including study of allosteric regulatory mechanisms, utilizing phosphodiesterase inhibitors.

Publicatons:

Manganiello, V.C., Yamamoto, T., Elks, M.L., Lin, M.C., and Vaughan, M.: Regulation of specific forms of cyclic nucleotide phosphodiesterases in cultured cells. In Strada, S.J. and Thompson, W.J. (Eds.): Advances in Cyclic Nucleotide Research. New York, Raven Press, 1984, Vol. 16, pp. 291-301.

Strewler, G.J., Danello, M.A., Manganiello, V.C., and Vaughan, M.: Ca^{2+}-independent cyclic GMP phosphodiesterases from rat liver and HTC hepatoma cells. Biochem. J. 213: 379-386, 1983.

Yamamoto, T., Manganiello, V.C., and Vaughan, M.: Purification and characterization of cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver: Effects of divalent cations on activity. J. Biol. Chem. 258: 12526-12533, 1983.

Yamamoto, T., Lieberman, F., Osborne, J.C., Jr., Manganiello, V.C., Vaughan, M., and Hidaka, H.: Selective inhibition of two soluble adenosine cyclic 3'5'-phosphate phosphodiesterases purified from calf liver. Biochemistry 23: 670-675, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00635-03 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Bordetella Pertussis Toxin on Adenylate Cyclase Inhibition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Judith A. Hsia, M.D.	Medical Staff Fellow	CM, NHLBI
Others:	Joel Moss, M.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Charlottesville, VA (E. L. Hewlett, M.D.)

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adenylate cyclase in many tissues is dually regulated, that is, subject to both stimulatory and inhibitory control. In eukaryotic cells, hormonal inhibition of adenylate cyclase is mediated by specific receptors and an inhibitory guanyl nucleotide-binding regulatory protein (Gi), whereas hormonal stimulation is mediated by receptors for stimulatory ligands and a stimulatory guanyl nucleotide-binding regulatory protein, Gs. Choleragen catalyzes the transfer of ADP-ribose from NAD to Gs which is thereby irreversibly activated. Bordetella pertussis toxin (PT) ADP-ribosylates Gi, abolishing the effects of inhibitory agonists and increasing basal adenylate cyclase activity. To study this apparently paradoxical stimulation of cyclase activity, NG108-15 (neuroblastoma x glioma hybrid) cells were incubated with PT or choleragen or with both toxins. Maximal cyclase activity observed following incubation with both toxins was greater than that seen following incubation with either toxin alone. This is consistent with the hypothesis that Gi exerts a basal inhibitory tone on the catalytic moiety which is relieved following ADP-ribosylation with PT. The amino acid acceptor for PT-catalyzed ADP-ribosylation is asparagine in transducin, a retinal protein structurally related to Gs and Gi. ADP-ribose-protein bonds have been described as stable or labile to neutral hydroxylamine. ADP-ribose-glutamate was thought to be the labile bond and ADP-ribose-arginine the stable bond. Study of the ADP-ribose-asparagine bond formed by pertussis toxin indicates that it represents a third class of ADP-ribose linkage which is even more stable to hydroxylamine degradation. The ADP-ribose-Gi linkage formed by PT is as stable as the ADP-ribose-transducin bond, suggesting that the PT substrate in Gi is asparagine. ADP-ribose bonds of similar stability are formed in membranes from human erythrocytes and NG108-15 cells after incubation with 32-P-NAD, suggesting the existence of tissue ADP-ribosyl-(asparagine)protein transferases in addition to the previously described ADP-ribosyl-(arginine)protein transferases.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: When many hormones bind to their target cells, the ligands' presence is signalled across the plasma membrane by adenylate cyclase. The structure of this enzyme is complex, consisting of a catalytic unit, specific hormone receptors, and distinct stimulatory and inhibitory GTP-binding regulatory proteins, called G_s and G_i, respectively. G_s and G_i are heterotrimers of α-, β-, and γ-subunits which dissociate into α- and $\beta\gamma$-subunits upon activation. The $\beta\gamma$-subunits are identical and α-subunits are structurally similar. Choleragen irreversibly activates G_s by ADP-ribosylating its α-subunit. Bordetella pertussis toxin (PT) catalyzes transfer of ADP-ribose from NAD to $G_{i\alpha}$, thereby abolishing hormonal inhibition of adenylate cyclase and increasing basal cyclase activity. It had been proposed by Katada et al. that G_i did not interact directly with the catalytic unit but affected enzymatic activity by absorbing the $\beta\gamma$-subunit from G_s, freeing $G_{s\alpha}$ to stimulate the catalytic unit. We studied G_i-catalytic unit interaction by investigating the ability of PT to stimulate catalytic activity after G_s had been fully activated by hormones or choleragen.

Transducin, a GTP-binding retinal protein which regulates the activity of a light-sensitive cGMP phosphodiesterase, is structurally related to G_s and G_i. PT ADP-ribosylates an asparagine residue in transducin. Other ADP-ribose-amino acid bonds have been characterized by their susceptibility to degradation by neutral hydroxylamine. We investigated the hydroxylamine sensitivity of the ADP-ribose-(asparagine)transducin bond and compared it to the ADP-ribose-G_i bond formed by PT. By studying the hydroxylamine sensitivity of ADP-ribose linkages formed in tissues without addition of PT, we sought evidence of endogenous ADP-ribosyl(asparagine)protein transferases which have not been previously described.

Methods Employed: Purified PT was provided by Dr. Erik L. Hewlett, University of Virginia. NG108-15 cells were incubated with choleragen or PT for various intervals before determining cAMP content or harvesting for membrane preparation. Membranes were assayed for basal adenylate cyclase activity, PGE_1 stimulation, and enkephalinamide inhibition. For hydroxylamine degradation experiments, membranes from NG108-15 cells, human fibroblasts or erythrocytes, bovine retinal rod outer segments, or purified G_i from bovine liver were ^{32}P-ADP-ribosylated with PT or choleragen. After incubation with neutral hydroxylamine or NaCl for various intervals, the amount of ^{32}P-ADP-ribose covalently linked to the proteins was quantified by SDS-polyacrylamide gel electrophoresis, autoradiography, and laser densitometry.

Major Findings: In membranes from NG108-15 cells, adenylate cyclase activity is increased 8-fold following incubation with choleragen (1 µg/ml, 2 h). Addition of PGE_1, a stimulatory hormone, to assays caused no significant further increase. Cyclase activity was reduced 22% by enkephalinamide, an opiate agonist, both in control and choleragen-treated cells. Following incubation with PT (40 ng/ml, 2 h), enkephalinamide inhibition was abolished and basal cyclase activity increased 50%. Incubation with both choleragen and PT resulted in a 13-fold increase in cyclase activity and enkephalinamide inhibition was abolished. Similarly, in the presence of Ro 20-1724, a phosphodiesterase inhibitor, cAMP content was increased 50 times by choleragen, 2 times by PT, and 70 times by both toxins. This synergistic stimulation of adenylate cyclase by choleragen and

PT suggests that G_i acts on the catalytic unit directly rather than having its effect only through G_s.

The ADP-ribose-asparagine bond formed by PT in transducin was stable to 0.5 M hydroxylamine, pH 7.5, for at least 2 h at 37°C. In contrast, the ADP-ribose-arginine linkage formed by choleragen in transducin was degraded with a half-life of 1 h. Thus, the ADP-ribose-asparagine bond represents a new category of ADP-ribose linkage with regard to hydroxylamine sensivity. Further, the ADP-ribose linkages formed in G_i (by PT) and G_s (by choleragen) are identical in stability to those formed in transducin by the two toxins, consistent with the probability that asparagine and arginine are modified in G_i and G_s, respectively. Hydroxylamine-stable bonds were found in membranes from various nonintoxicated mammalian cells following incubation with ^{32}P-NAD, which may reflect the presence of tissue NAD: protein-ADP-ribosyltransferases other than the previously described transferases that use arginine and glutamate as acceptors. Attempts to identify asparagine-transferases using asparagine as a model acceptor were unsuccessful, presumably because protein conformation is crucial to formation of ADP-ribose-asparagine; PT displays marked substrate specificity. Identifying tissue substrates by their hydroxylamine resistance would provide a means for identifying and purifying these putative enzymes.

Significance to Biomedical Research and the Program of the Institute: Adenylate cyclase is a ubiquitous enzyme that plays a crucial role in mediating the actions of a variety of hormones and drugs. Our study has provided new information about its regulation and has suggested a means for identification and purification of endogenous tissue NAD: ADP-ribosyltransferases that, like PT, modify specific asparagine residues in protein.

Proposed Course: Dr. Hsia's appointment terminated June 1984.

Publications:

Hsia, J. A., Moss, J., Hewlett, E. L., and Vaughan, M.: ADP-ribosylation of adenylate cyclase by pertussis toxin: Effects on inhibitory agonist binding. J. Biol. Chem. 259: 1086-1090, 1984.

Moss, J., Stanley, S. J., Burns, D. L., Hsia, J. A., Yost, D. A., Myers, G. A., and Hewlett, E. L.: Activation by thiol of the latent NAD-glycohydrolase and ADP-ribosyltransferase activities of Bordetella pertussis toxin (islet-activating protein). J. Biol. Chem. 258: 11879-11882, 1983.

Hsia, J. A., Moss, J., Hewlett, E. L., and Vaughan, M.: Requirement for both choleragen and pertussis toxin to obtain maximal activation of adenylate cyclase in cultured cells. Biochem. Biophys. Res. Commun. 119: 1068-1074, 1984.

Moss, J., Bruni, P., Hsia, J. A., Tsai, S.-C., Watkins, P. A., Halpern, J. L., Burns, D. L., Kanaho, Y., Chang, P. P., Hewlett, E. L., and Vaughan, M.: Pertussis toxin catalyzed ADP-ribosylation: Effects on the coupling of inhibitory receptors to the adenylate cyclase system. J. Receptor Res., in press.

Hsia, J. A.: Pertussis toxin: Toxin-catalyzed ADP-ribosylation blocks hormonal inhibition of adenylate cyclase. In: Moss, J., moderator. Cyclic nucleotides: mediators of bacterial toxin action in disease. Ann. Intern. Med., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00636-03 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Regulation of Particulate cAMP Phosphodiesterase in 3T3-L1 Fatty Fibroblasts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)*

PI:	Martha L. Elks, M.D., Ph.D.	Medical Staff Fellow	CM, NHLBI
OTHERS:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

Differentiated 3T3-L1 adipocytes were used to study the control and metabolic role of cellular phosphodiesterases. Incubation of these cells with 100 pM insulin or 1 μM isoproterenol resulted in activation of the particulate cAMP phosphodiesterase within 10 min. The effect of insulin but not that of isoproterenol was prevented by prior treatment of cells with pertussis toxin.

In cells maintained for 2 weeks in hypothyroid medium (medium with 10% serum from a hypothyroid calf), basal lipolysis and cAMP levels and sensitivity to isoproterenol stimulation were decreased. Opposite changes in lipolysis and cAMP levels occurred in cells maintained in hyperthyroid medium. In hypothyroid cells, particulate and soluble cAMP phosphodiesterase activity was increased. Sensitivity of adenyl cyclase in these cells to stimulation by isoproterenol was decreased, but there was no change in β-adrenergic receptor number or affinity. Conversely, in hyperthyroid cells, particulate and soluble cAMP phosphodiesterases were decreased; adenyl cyclase activity and sensitivity to stimulation by isoproterenol were increased, but there was no change in β-receptor number or affinity. The change in hormone-stimulated lipolysis observed in different thyroid states in these cells may be secondary to changes in both phosphodiesterase activity and receptor-cyclase coupling.

The soluble cAMP phosphodiesterase activity in these cells is more sensitive to inhibition by Ro 20-1724, but the particulate activity is more sensitive to inhibition by cilostamide. In cells incubated with cilostamide, stimulated cAMP levels were lower, but lipolysis (basal and isoproterenol sensitivity) was greater than in cells incubated with Ro 20-1724. These findings suggest that the particulate cAMP phosphodiesterase may be important in the metabolism of cAMP involved in the regulation of lipolysis.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives: To define mechanisms for control of cAMP phosphodiesterase activity, cAMP content, and lipolysis in 3T3-L1 adipocytes including effects of thyroid hormone, phosphodiesterase inhibitors and other agents.

Methods Employed: 3T3-L1 fibroblasts, obtained from Dr. M. D. Lane of Johns Hopkins University, were grown to confluence in six-well culture plates. Two days later, cells were treated with 1 μM dexamethasone, 1 μM insulin, and 0.1 mM methylisobutylxanthine (IBMX) for 72 hr, then incubated in fresh growth medium. Within a week cells developed characteristics of mature adipocytes. At stages in development of the adipocyte phenotype, cells were exposed to hormones or drugs, harvested by scraping, and homogenized. After centrifugation (100,000 x g, 45 min), samples of supernatant or particulate fractions were assayed for cAMP phosphodiesterase activities. Lipolysis was measured as glycerol production in Hanks' medium with bovine serum albumin, 10 mg/ml. ^{125}I-hydroxybenzylpindolol binding was quantified in a filtration assay.

Major Findings: Differentiation of 3T3-L1 fibroblasts, monitored by accumulation of neutral lipid and increase in α-glycerophosphate dehydrogenase activity, is accelerated by incubation of confluent cells with insulin, dexamethasone, and IBMX. IBMX inhibits cyclic nucleotide phosphodiesterases as well as the binding of adenosine to its receptor. Agents with relatively specific effects were utilized to examine the role of IBMX in differentiation. Ro 20-1724, a selective inhibitor of soluble cAMP phosphodiesterase activities, was as effective as IBMX in increasing α-glycerophosphate dehydrogenase activity and fat deposition. Neither cilostamide, which inhibits particulate but not soluble cAMP phosphodiesterase activities, 8-phenyltheophylline, an adenosine receptor antagonist with little inhibitory effect on phosphodiesterase activities, nor N^6-(R phenyl-isopropyl) adenosine (PIA), a potent adenosine receptor agonist, promoted differentiation. Maximal increases in α-glycerophosphate dehydrogenase activity and lipid accumulation were observed when differentiation was initiated in the presence of 10 nM dexamethasone. These data suggest that inhibition of soluble cAMP phosphodiesterase activity and subsequent alterations in cAMP may be important in the mechanism whereby IBMX enhances differentiation of 3T3-L1 cells.

Incubation of adipocytes with insulin or isoproterenol for 10 min increases particulate "low K_m" cAMP phosphodiesterase activity. Pertussis toxin catalyzed the [^{32}P]ADP-ribosylation of a 41,000 dalton protein in adipocyte particulate fractions; prior incubation of adipocytes with toxin markedly reduced incorporation of radiolabel. Exposure of adipocytes to pertussis toxin (0.3 μg, 18 hr) increased glycerol production and inhibited activation of cAMP phosphodiesterase by insulin but not by isoproterenol. These results suggest that pertussis toxin can interfere with receptor-mediated processes that stimulate cAMP hydrolysis as well as those that inhibit cAMP formation.

Particulate cAMP phosphodiesterase activity from adipocytes was very sensitive to inhibition by cilostamide in an apparently competitive fashion. Particulate activity from undifferentiated fibroblasts or supernatant activity from either type of cell was much less sensitive to cilostamide. On the other hand, supernatant cAMP phosphodiesterase activity from both undifferentiated fibroblasts and adipocytes was very sensitive to inhibition by Ro 20-1724 in an apparently

competitive fashion. Ro 20-1724 was not an effective inhibitor of particulate activity from either type of cell. In fractions from 3T3-L1 adipocytes, IBMX inhibited both supernatant and particulate cAMP phosphodiesterase activities. IBMX was relatively more specific in inhibiting supernatant calmodulin-activated cGMP phosphodiesterase activity than supernatant calmodulin-independent or particulate cGMP phosphodiesterase activities.

In intact adipocytes, cilostamide enhanced lipolysis with or without isoproterenol and had no effect on cAMP content in the presence of low concentrations of isoproterenol. Ro 20-1724 increased isoproterenol-stimulated accumulation of cAMP to a greater extent than cilostamide but had a smaller effect on basal and isoproterenol-stimulated lipolysis. Like cilostamide, Ro 20-1724 did not enhance lipolysis or cAMP accumulation in the presence of IBMX with or without isoproterenol. Taken together these results support the idea that, although particulate and soluble "low K_m" phosphodiesterases both influence cAMP content, the particulate enzyme may be more important in the metabolism of cAMP involved in regulation of lipolysis. Since combinations of Ro 20-1724 and cilostamide were not as effective as IBMX in increasing cAMP content, perhaps the calmodulin-dependent phosphodiesterase, which is selectively inhibited by IBMX, is also involved in the regulation of total cell cAMP content.

Effects of triiodothyronine on β-adrenergic regulation of lipolysis, adenylate cyclase, and phosphodiesterase were investigated. Differentiated adipocytes were maintained in four media: 1) medium containing serum from a hypothyroid calf (hypothyroid cells), 2) hypothyroid medium supplemented with triiodothyronine to a normal level, 3) medium with serum from a normal calf, and 4) medium with normal serum supplemented with excess T_3 (hyperthyroid cells). Compared to control groups (2 and 3), hypothyroid cells exhibited lower basal rates of lipolysis and reduced sensitivity to isoproterenol. Hyperthyroid cells exhibited higher basal rates of lipolysis and increased sensitivity to isoproterenol. With maximal isoproterenol stimulation, rates of lipolysis were similar in the four groups. Basal cAMP content and cAMP accumulation in the presence of isoproterenol were reduced in hypothyroid and increased in hyperthyroid cells as compared to controls. Basal adenylate cyclase activity was similar in the four groups. Sensitivity to isoproterenol and maximal isoproterenol-stimulated cyclase activity were diminished in membranes from hypothyroid and increased in preparations from hyperthyroid cells. NaF-stimulated activity was increased in preparations from hyperthyroid cells; hypothyroid cells were similar to controls. Thyroid status did not affect β-receptor number or affinity for iodohydroxybenzylpindolol. Both soluble and particulate cAMP phosphodiesterase activities were increased in hypothyroid and decreased in hyperthyroid cells compared to control groups.

These results indicated that in 3T3-L1 adipocytes some of the effects of thyroid hormone on cAMP content and lipolysis can be explained by alterations in both production and degradation of cAMP.

Significance to Biomedical Research and the Program of the Institute: cAMP is an important messenger in the control of multiple metabolic processes in virtually all mammalian cells. Local availability of cAMP is dependent upon the rate of its production by adenylate cyclase and its degradation by phosphodiesterases. Thus, regulation of activity of these enzymes is central to the mechanisms through which many hormones control cellular function. Our findings

suggest that regulation of lipolysis by glucocorticoid and thyroid hormones may be in part accounted for by alterations in both adenylate cyclase and diesterase systems. Furthermore, our data suggest that a specific phosphodiesterase may be important in the metabolism of cAMP involved in the regulation of lipolysis.

Proposed Course: Further investigation of the mechanism of activation of particulate cAMP phosphodiesterase by insulin and its prevention by pertussis toxin as well as the role of this phosphodiesterase in the antilipolytic activity of insulin.

Publications:

Elks, M.L., Watkins, P.A., Manganiello, V.C., Moss, J., Hewlett, E., and Vaughan M.: Selective regulation by pertussis toxin of insulin-induced activation of particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes. Biochem. Biophys. Res. Commun. 116: 593-598, 1983.

Elks, M.L., Manganiello, V.C., and Vaughan, M.: Effect of dexamethasone on cAMP content and phosphodiesterasse activities in 3T3-L1 adipocytes. Endocrinology, in press.

Elks, M.L. and Manganiello, V.C.: Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, cAMP metabolism, and lipolysis in 3T3-L1 adipocytes. Endocrinology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00638-02 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transducin GTPase: Genes for GTP-Binding Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Drusilla L. Burns, Ph.D.	Staff Fellow	CM, NHLBI
Others:	C. William Angus, Ph.D.	Staff Fellow	CM, NHLBI
	Jane L. Halpern, Ph.D.	Staff Fellow	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.9	2.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

GTP-binding proteins often play critical roles in cellular metabolism. Three GTP-binding regulatory proteins are termed Gs, Gi, and transducin which mediate hormonal stimulation of adenylate cyclase, hormonal inhibition of adenylate cyclase, and light activation of the phosphodiesterase of retinal rod outer segments, respectively. All three proteins exhibit striking structural and functional homologies. In order to define the molecular mechanisms by which these proteins mediate hormonal or light-controlled processes, we have begun to clone the genes coding for these proteins.

Since transducin can be isolated in larger quantities than Gs or Gi, initial studies were directed towards purifying transducin subunits. After isolation of transducin and separation of its three subunits (termed α, β, and γ), sequence data from each of the subunits were obtained. Oligonucleotide probes were synthesized based on these sequences. In addition, polyclonal and monoclonal antibodies to holotransducin and its subunits were prepared to use as additional probes for the transducin genes. These antibodies were found to interfere with transducin enzymatic activity. Moreover, antitransducin $\beta\gamma$ antibodies cross-reacted with the corresponding adenylate cyclase components.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: Several GTP-binding proteins are important regulators of metabolic processes. Three of these GTP-binding proteins are termed G_s, G_i, and transducin. G_s and G_i mediate hormonal stimulation and inhibition of adenylate cyclase activity. Transducin regulates the light-activated phosphodiesterase of retinal rod outer segments. G_s, G_i, and transducin each consist of three subunits designated α, β, and γ. The $\beta\gamma$ subunits of G_s and G_i appear to be identical in size and structure and are similar to the $\beta\gamma$ subunits of transducin. The α subunits of G_i and transducin also exhibit striking homologies. In order to better understand the molecular basis by which these proteins regulate metabolic processes, we have begun to clone the genes coding for these proteins. Knowledge of the structure of these genes will provide information concerning the structure and regulation of these proteins and will facilitate studies aimed at understanding the function of these proteins. Intermediate objectives, therefore, include purification of proteins for sequence determination, preparation of oligonucleotide probes, and production of monoclonal and polyclonal antibodies.

Methods Employe: Transducin was prepared as previously described (Watkins, P.A., Moss, J., Burns, D.L., Hewlett, E.L., and Vaughan, M., J. Biol. Chem. 259: 1378-1381, 1984). The α and $\beta\gamma$ subunits were separated using Blue Sepharose CL-6B (Shinozawa, T., Uchida, S., Martin, E., Cafiso, D., Hubbell, W., and Bitensky, M., Proc. Natl. Acad. Sci. U.S.A. 77: 1408-1411, 1980).

Antibodies to transducin were prepared by injection of rabbits with holotransducin or transducin α and $\beta\gamma$ subunits; antisera (titer > 1/1000) reacted with all subunits. Monoclonal antibodies were produced using Balb/c mice and SP 2/0 myeloma cells. Antibodies were quantified by ELISA.

Major Findings: (1) Polyclonal and Monoclonal Antibodies to Transducin and the Guanyl Nucleotide-Binding Proteins of Adenylate Cyclase. Polyclonal antibodies were prepared in rabbits to bovine retinal transducin and to $\text{transducin}_{\alpha}$ and $\text{transducin}_{\beta\gamma}$. Since the amino acid composition and protease digestion patterns of the α and β transducin and G_i are similar, the transducin antibodies were screened by Western blots for cross-reactivity with the G_i subunits purified from rat liver. Antibodies against the $\beta\gamma$ subunits of transducin recognized the 35 kDa subunit of G_i but not the 10k subunit. Antibodies against the 39 kDa subunit of transducin did not recognize the 41 kDa subunit of G_i. One monoclonal antibody of isotype IgG_{2A} specific for T_{α} has been purified and partially characterized. When the antibody was incubated with transducin subunits and rhodopsin in a reconstituted system, it inhibited the GTPase activity of transducin as well as Gpp(NH)p binding.

(2) Cloning of the Genes for the Adenylate Cyclase and Rhodopsin-Transducin Systems: The inhibitory guanyl nucleotide-binding component of the adenylate cyclase system, termed G_i, appears to be structurally and functionally similar to transducin, the coupling protein in the rhodopsin-phosphodiesterase system. Since the rhodopsin-transducin components can be isolated in higher yield and quantities than those of adenylate cyclase, initial studies are directed at obtaining sequence data on transducin and using that information to synthesize oligonucleotide probes; the probes will then be used to isolate cDNA clones.

No amino acid sequence information about the transducin subunits was available at the time this project was begun; therefore, the partial amino acid sequence of each subunit was determined. Transducin is composed of three subunits termed α, β, and γ which have molecular weights of 39,000, 35,000, and 10,000, respectively. Each subunit was isolated and subjected to automated Edman degradation. In this manner, the N-terminal 26 amino acids of the γ subunit were determined. No sequence was obtained for either α or β subunits, indicating that their N-terminal amino acids were blocked. Fragments of the α and β subunits were, therefore, prepared by proteolysis and partial acid hydrolysis. One fragment of the α subunit and two fragments of the β subunit were sequenced, yielding information from which suitable oligonucleotide probes are being synthesized.

Significance to Biomedical Research and the Program of the Institute: Adenylate cyclase plays a critical role in many tissues including the heart and lung by mediating the actions of a number of hormones. Transducin plays a similar regulatory role in retinal rod outer segments by mediating light activation of a cGMP phosphodiesterase. These studies will aid in the understanding of the structures, regulation, and functions of these GTP-binding proteins.

Proposed Course: Continued acquisition of protein sequence data, preparation of oligonucleotide probes, and characterization of antibodies to facilitate isolation of the genes coding for G_s, G_i, and transducin.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00639-01 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of a Bovine Rod Outer Segment cGMP Phosphodiesterase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
Others:	Drusilla L. Burns, Ph.D.	Staff Fellow	CM, NHLBI
	Martha L. Elks, M.D., Ph.D.	Medical Staff Fellow	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Hideo Wada, M.D., Ph.D.	Visiting Fellow	CM, NHLBI
	Paul A. Watkins, M.D., Ph.D.	Medical Staff Fellow	CM, NHLBI
	Martha Vaughan, M.D.	Chief	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	0.8	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cGMP phosphodiesterase was extracted from bovine rod outer segments by suspension of rod outer segments in hypotonic medium. Phosphodiesterase was further purified by chromatography on AcA 34; polyacrylamide gel electrophoresis in SDS indicated that the holoenzyme was comprised of 3 subunits (~ 88, 86, and 10 kDa). Enzyme activity was markedly increased by trypsin; activity was also increased severalfuld in the presence of NAD and a mono-ADP-ribosyltransferase from turkey erythrocytes. Incubation of the phosphodiesterase with the transferase and [^{32}P]NAD indicated that both high and low molecular weight subunits were ADP-ribosylated.

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Project Description:

Objectives: To characterize regulatory properties of the light-activated cGMP phosphodiesterase (PDE) from bovine rod outer segments (ROS) and effects of limited proteolysis and covalent modification on activity.

Methods Employed: Purification of enzyme. Hypotonic extracts from ROS were concentrated and chromatographed on AcA 34 columns equilibrated and eluted with 50 mM Hepes, pH 7.4/0.4 M NaCl/1.0 mM EGTA/1 mM NaN_3 plus protease inhibitors leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. Purity was assessed by polyacrylamide slab gel electrophoresis in SDS.

Major Findings: An M_r of ~ 196,000 for the purified PDE was estimated from sedimentation equilibrium data; gel electrophoresis under denaturing conditions showed three subunits of ~ 88, 86, and 10k Da. Proteolysis of the PDE with trypsin or chymotrypsin increased activity (V_{max}). Incubation of purified PDE for 30 min at 30°C with a purified ADP-ribosyltransferase from turkey erythrocytes and NAD also caused activation (but not to the extent achieved with trypsin). In the presence of [^{32}P]NAD and the transferase, both high and low molecular weight subunits of the PDE were labeled. After maximal activation of PDE with trypsin, further incubation with ADP-ribosyltransferase and NAD decreased activity. Incubation of ROS with the transferase and NAD increased PDE activity. The role of ADP-ribosylation in physiological regulation of PDE activity remains to be determined, although it is clear that this covalent modification can alter behavior of the enzyme in intact ROS as well as after purification.

Significance to Biomedical Research and the Program of the Institute: This phosphodiesterase, which plays a major role in the process of visual excitation, is controlled through a receptor/GTP-binding protein system analogous to that of the hormone-sensitive adenylate cyclase. Thus, information about this enzyme may aid in our understanding the cyclase system as well as the properties of other cyclic nucleotide phosphodiesterases (especially the hormone-sensitive particulate phosphodiesterase in adipose tissue) which are major targets of the LCM research program.

Proposed Course: Characterization of this enzyme with emphasis on reconstitution studies with purified regulatory components of the light-sensitive phosphodiesterase system as well as the hormone-sensitive phosphodiesterase and adenylate cyclase systems.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00640-01 CM

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of the p-Nitrophenyl Phosphatase Activity of Calcineurin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Sharon J. Geyer, Ph.D.	Guest Worker	CM, NHLBI
OTHERS:	Randall L. Kincaid, Ph.D.	Research Pharmacologist	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS (if any)

Muscular Dystrophy Association, New York, NY

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcineurin, a calmodulin-binding protein, dephosphorylates several phosphoproteins as well as phosphotyrosine and p-nitrophenyl phosphate. Some charactristics of the p-nitrophenyl phosphate activity of calcineurin purified from bovine brain were investigated. In standard assays with 100 mM substrate, 0.5 mM MnCl-2 and 0.8 mM CaCl-2, calmodulin increased phosphatase activity 150-300%. Activity was largely dependent on Mn2+; omission of Ca2+ decreased activity < 15% with or without Ca2+-free calmodulin, whereas omission of Mn2+ decreased activity > 90%. When Ni2+ (1-10 mM) was substituted for Mn2+, activity was increased ~ 20%. However, with Mn2+ present, Ni2+ did not increase activity. The effect of calmodulin on calcineurin activity was dependent on substrate concentrations. Lineweaver-Burk plots were nonlinear with and without calmodulin. At p-nitrophenyl phosphate concentrations up to 50 mM, calmodulin increased maximal velocity and had little effect on apparent Km (~ 30 mM); at > 50 mM substrate, calmodulin decreased the apparent Km but did not appreciably alter Vmax. With 0.1 μM calcineurin, the same concentration of calmodulin produced maximal activation, indicating that the active complex is a heterotrimer. Calmodulin antagonists trifluoperazine, W-7, and W-5 inhibited calmodulin-dependent phosphatase activity; the concentrations required were 2-3 times those necessary for inhibition of calmodulin-dependent phosphodiesterase activity. Inhibition of basal and calmodulin-dependent phosphatase activity by orthovanadate (Ki 0.5 μM) is consistent with other evidence that calcineurin may be a phosphotyrosyl-protein phosphatase. Dimethylsulfoxide (30%) increased activity 100% above that observed with maximal calmodulin activation. Thus, calmodulin, at least under the assay conditions employed, apparently does not permit full expression of catalytic activity.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: Calcineurin, the predominant calmodulin-binding protein in brain, is reportedly identical to protein phosphatase 2B from skeletal muscle which is activated by calmodulin. Calcineurin phosphatase activity with several phosphoproteins as well as phosphotyrosine and p-nitrophenyl phosphate has been reported. The natural substrate(s) is unknown. Effects of divalent cations (e.g., Ca^{2+}, Mn^{2+}, Ni^{2+}) on activity apparently differ with different substrates and/or calcineurin preparations. To define the regulatory properties of calcineurin and the mechanism of calmodulin activation, we have used p-nitrophenyl phosphate as a model substrate.

Methods Employed: Calcineurin and calmodulin purified from bovine brain by methods developed in this laboratory were > 95% pure based on Coomassie blue staining of SDS gels. Calcineurin preparations contained equivalent amounts of 61 and 18 kDa subunits. Hydrolysis of p-nitrophenyl phosphate was measured spectrophotometrically.

Major Findings: In standard assays with 0.8 mM $CaCl_2$ and 0.5 mM $MnCl_2$, calmodulin increased phosphatase activity ~ 4-fold. Omission of Ca^{2+} decreased activity < 10% with or without calmodulin, i.e., the degree of calmodulin stimulation was essentially unchanged. When Mn^{2+} was omitted, activity was decreased > 90% and was even lower when both Mn^{2+} and Ca^{2+} were omitted. Without Ca^{2+}, the concentration of $MnCl_2$ required for half-maximal activity was 50 μM in the absence and 90 μM in the presence of calmodulin. In standard assays, $NiCl_2$ at concentrations up to 1 mM had little effect; > 5 mM $NiCl_2$ increased basal activity slightly and inhibited calmodulin-dependent activity. In assays without Mn^{2+}, Ni^{2+} markedly increased phosphatase activity with and without calmodulin; 0.5-1 mM $NiCl_2$ produced half-maximal effects. Maximal activities observed with 5-10 mM $NiCl_2$ were 10-20% greater than those with optimal Mn^{2+}.

Activity was maximal between pH 8.0 and 8.5. Lineweaver-Burk plots for p-nitrophenyl phosphate were nonlinear with and without calmodulin. With substrate concentrations below 50 mM, calmodulin increased maximal velocity and had little effect on K_m (29-33 mM). With > 50 mM substrate, it appeared that calmodulin decreased the K_m (from ~ 200 mM) but did not increase maximal velocity.

Maximal activation of 0.1 μM calcineurin was achieved with equimolar calmodulin, in agreement with the stoichiometry of the calcineurin-dansyl-calmodulin interaction shown in this laboratory and with the conclusion that the active complex is a heterotrimer. Inhibition of calmodulin-dependent calcineurin activity required somewhat higher concentrations of trifluoperazine, W-7, and W-5 than were necessary for inhibition of calmodulin-dependent phosphodiesterase activity. Whether this is due to differences in the conditions for assay of the two enzymes or to differences in their interactions with calmodulin is unclear.

Orthovanadate was a potent inhibitor of calcineurin phosphatase (K_i 0.5 μM). This observation, along with the inhibition by Zn^{2+} (80-90% with 10 μM) and insensitivity to fluoride (up to 10 mM), is consistent with the possibility that calcineurin is a phosphotyrosyl-protein phosphatase.

Calcineurin activity in the presence of 30% dimethylsulfoxide was substantially greater than that observed with calmodulin. Thus, it appears that, under our standard assay conditions, even with apparently maximal activation by calmodulin (and Mn^{2+}), catalytic activity is not fully expressed. By elucidating the mechanism of activation by dimethylsulfoxide, it may be possible to learn more about the regulatory properties of calcineurin.

Significance to Biomedical Research and the Program of the Institute: Calmodulin and Ca^{2+} have significant roles in regulating numerous cell functions including muscle contraction and glycogen metabolism. Regulation by Ca^{2+} and/or calmodulin in part depends on their ability to affect the activity of protein kinases as well as the protein phosphatase activity of calcineurin. Characterization of the enzymatic properties of calcineurin will provide new information about the dual function of Ca/calmodulin in regulating protein phosphorylation/dephosphorylation.

Proposed Course: Recent observations in our laboratory and others have shown that calcineurin is deactivated as well as activated by calmodulin plus Ca^{2+}. Investigation of effects of divalent cations on deactivation and reactivation as well as attempts to delineate the mechanism of activation by dimethylsulfoxide and similar effectors are in progress.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMICAL PHARMACOLOGY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 to September 30, 1984

In the past, this Laboratory has developed several approaches for discovering drugs and other foreign compounds that cause tissue lesions through the formation of chemically unstable metabolites. In recent years, it has focused its attention on discovering the identity of the toxic metabolites and the factors that govern the relative severity of the toxicity in different target organs of different species and strains of animals.

Although the Laboratory has continued along these lines of research during the the past year, it has shifted its emphasis in several ways. Most chemically reactive metabolites are formed by several enzymes that are collectively known as cytochrome P-450. Although many of the isozymes of cytochrome P-450 have been isolated and in some instances their amino acid sequence established, there is no evidence that the enzymatic activities of the purified forms in reconstituted systems mimic exactly their activities in liver microsomes or in vivo. The Laboratory, therefore, is developing approaches by which the substrate specificity of a given isozyme of cytochrome P-450 may be assessed in the presence of other forms of cytochrome P-450 within microsomes.

Since chemically reactive metabolites may evoke their toxicities through several diverse mechanisms, the Laboratory is also exploring several ways by which such metabolites might evoke toxicities.

Formation and Decomposition of Unstable Metabolites

Bromobenzene metabolism. Several years ago Reid in our Laboratory found that bromobenzene in rats caused cellular necrosis not only in liver but also in lung and kidney. Two years ago we established that liver necrosis was not caused by p-bromophenol, or o-bromophenol or 4-bromocatechol even in doses equivalent to the bromobenzene doses used by Reid. Thus, the liver necrosis caused by bromobenzene is apparently mediated by bromobenzene-3,4-oxide as suggested by Reid. The mechanism by which bromobenzene evoked extrahepatic toxicity, however, remained to be established.

Two years ago, we discovered that bromobenzene-3,4-oxide was sufficiently stable to escape hepatocytes and to enter the extracellular medium where it can be trapped as material covalently bound to extracellular glutathionyl transferase B. Because some of the epoxide reacted with extracellular glutathione, however, the amount that was covalently bound to the transferase represented a minimal estimate. By using radiolabeled glutathione, we could also estimate the fraction of the glutathione conjugates that were formed extracellularly. With these estimates, we now calculate that about 15% of the epoxide is converted to glutathione conjugates intracellularly as the epoxide is formed and that about 85% escapes the cells and decomposes in the medium to various metabolites including 3,4-dihydrobromobenzene-3,4-diol. This was surprising, because it suggests that very little of the dihydrodiol was formed enzymatically.

By a similar approach, we were able to detect indirectly the presence of bromobenzene-3,4-oxide in venous blood of rats treated with bromobenzene. We were also able to determine that its half-life in blood was about 13.5 seconds, which would be sufficiently long to permit the epoxide to reach virtually every cell in the body.

Although bromobenzene-3,4-oxide formed in liver may contribute to extrahepatic toxicity, it certainly is not the sole toxicant. Two years ago we reported that in unpretreated rats o-bromophenol was five times more potent as a renal toxicant than was bromobenzene. During the past year, we found that o-bromophenol could be converted to bromohydroquinone and that bromohydroquinone in turn could be oxidized to a quinone which reacted with glutathione to form several glutathione conjugates. We further found that diglutathionyl bromohydroquinone was by far the most potent renal toxicant of the series and that the toxicity could be decreased by AT-125, which inhibits -glutamyl transpeptidase. In accord with these findings a mixture of cysteinyl derivatives of bromohydroquinone but not a mixture of N-acetyl cysteinyl derivatives of bromohydroquinone was nephrotoxic. Since cysteinyl derivatives are metabolized by β-lyases, it seems likely that the nephrotoxicity caused by bromobenzene is predominantly due either to a suicide inhibition of β-lyase in kidney caused by the cysteinyl derivatives or to the toxic effects of one or more of the thiol metabolites formed from the derivatives.

Chloroform - In previous years, we have raised the possibility that the necrosis found in proximal tubules of kidney caused by chloroform might occur by way of the formation of hydroxytrichloromethane which decomposes to phosgene. In accord with this hypothesis the severity of the toxicity paralleled the magnitude of the decrease in renal glutathione in rats, DBA male mice and C57 mice. Moreover, $CHCl_3$ was more potent than $CDCl_3$ as a renal toxicant and cause a greater decrease in renal glutathione. Last year we reported that chloroform was converted to phosgene by kidney homogenates and that differences in enzyme activity between the sexes of mice and differences in the isotope effect on metabolism of chloroform paralleled differences in the incidence of toxicities. During the past year we have extended these studies by demonstrating that differences between the renal enzyme activities in male DBA/2J and male C57/BL also paralleled the strain differences in the toxicity. Moreover, the activity of the enzyme in female ICR mice could be markedly increased by administration of testosterone.

Mechanisms of Toxicity

Halothane. It is now known that halothane may be converted to chemically reactive metabolites either by reductive cleavage of a carbon-halogen bond to form a free radical or by hydroxylation of the carbon-hydrogen bond followed by dehydrohalogenation to form a trifluoroacetyl halide. Although an acute hepatotoxicity has been shown to be associated with the reductive pathway, there is evidence that the fulminant type of halothane hepatotoxicity observed in humans may be due to a hypersensitivity reaction. We reasoned, however, that if a metabolite of halothane were to serve as a hapten in humans, the reactive intermediate would have to escape hepatocytes and become bound externally to plasma membranes. To demonstrate this possibility we developed an antibody which binds preferentially N-trifluoroacetyl lysine. With this antibody and

a fluorescent anti-IgG antibody we were able to show that halothane administered to rats becomes bound to the surface of hepatocytes. Moreover, with the antibody and a horseradish peroxidase conjugate of anti IgG we were able to show that binding occurred perferentially within the centrilobular zones of liver and that more binding occurred with CF_3-CClBrH than with CF_3CClBrD. Since only the hydroxylation reaction can account for this isotope effect, it is probable that most of the covalently bound metabolite arises from the trifluoroacetyl halide. Thus the mechanism of activation of halothane is analogous to that of chloroform.

Carbon tetrachloride. Carbon tetrachloride and other substances that promote lipid peroxidation in cells results in the destruction of cytochrome P-450 by causing the destruction of heme. During the past year, we have discovered not only that the heme is converted to a minor extent to water soluble fragments but that most of the heme decomposition products become covalently bound to cytochrome P-450. However, some of the heme fragments become covalently bound to proteins of very large molecular weight, which suggest that the heme fragments may link cytochrome P-450's with each other or with other microsomal proteins. In accord with this view, electrophoresis studies revealed that carbon tetrachloride in vitro causes a decrease in the amount of protein in the 48,000 to 60,000 range of molecular weights, a range that includes the molecular weights of the cytochrome P-450's. The possibility that most of the covalent binding of heme products to cytochrome P-450 may occur at the active site of the cytochromes P-450 is being explored.

Cardiotoxicity caused by analogues of adriamycin. Last year we reported that the toxic effects of adriamycin and daunamycin in cultured cardiomyocytes was apparently not due to lipid peroxidation initiated by superoxide and hydrogen peroxide but was associated with marked decreases in ATP and creatine phosphate. Electron micrographs have confirmed that the initial damage occurs into mitochondria. Working on the hypothesis that such marked alterations in ATP might might result from increases in intracellular concentrations of unbound calcium, we tested the effects of several substances on the severity of the toxicity. Verapamil potentiated the toxicity but nifedepine had no effect. However, amrinone and millrinone, which are ionotropes, prevented the toxicity caused by daunomycin.

Multiproduct Formation by Different Isozymes of Cytochrome P-450

In last year's report we pointed out that a monoclonal antibody developed by H. Gelboin's Laboratory (NCI) against cytochrome P-450c reacted with at least two isozymes in rat liver microsomes. During the past year, we isolated two isozymes that reacted with the antibody and that appeared to account for most, if not all, of the antibody sensitive isozymes in rat liver microsomes. The two isozymes reconstituted systems formed the major metabolites of R-propranolol at nearly identical relative rates quite similar to each other, but these relative rates were markedly different from the relative rates of formation of the metabolites by the antibody sensitive forms within liver microsomes. The differences do not appear to be due to the presence of an unidentified cytochrome P-450, because the relative rates of formation of the metabolites by the antibody sensitive enzymes in liver, kidney, lung and small intestines are remarkedly similar even though there are undoubtedly marked differences in the relative concentrations of the various isozymes of cytochrome P-450 in these organs. Together with other findings obtained in the Laboratory, it now appears that the conformations of the

active sites of the isozymes probably undergo alterations during purification. Such changes raise serious doubts about the validity of using reconstitution systems for estimating the relative contributions of various isozymes to the metabolism of drugs by liver microsomes and in vivo.

During course of these studies, we discovered that a glutathione conjugate could be formed from one of the intermediate metabolites of R-propranolol. In the presence of the soluble fraction the addition of glutathione led to the formation of the glutathione conjugate at the expense of 5-hydroxy propranolol but not at the expense of 4-hydroxypropranolol. These findings thus suggest that the 5-hydroxypropranolol is formed by way of R-propranolol-5, 6-oxide and that R-propranolol-3,4-oxide either is not formed, rearranges very rapidly or does not serve as a substrate for the glutathione-S-transferases.

Monoclonal antibodies. The Laboratory has made several monoclonal antibodies against purified cytochrome P-450. Their specificity is being evaluated.

Mechanism of Histamine Metabolism and Action

Release of histamine and other vasoactive mediators from tissue mast cells and blood basophils is the primary event in a variety of acute allergic and inflammatory conditions. During the past several years our objectives have been to characterize 1) histamine-containing cells in different tissues, 2) the sites of inactivation of the major mast cell constituent, histamine and 3) the types of responses mediated by different mast cell constituents in response to various allergic and inflammatory stimuli. These items are discussed separately below.

1) Biological characteristics of mast cells. Our focus has shifted from comparison of biochemical and morphological characteristics of histamine-containing cells in different tissues to studies of mechanisms of histamine synthesis, storage and release from mast cells. This year we have obtained specific information about these events in a rat basophilic leukemia (2H3) cells line. As with normal mast cells, uptake of the histamine precursor, histidine, is mediated by a temperature dependent system with high affinity (K_m = 24μM) for histidine that permits a concentration gradient (cell to medium ratio of 7:1). Uptake is inhibited competitively by histidine analogs and glutamine which permit further characterization of the uptake system. α-Fluoromethylhistidine, which selectively inactivates histidine decarboxylase by covalent binding, is taken up by the same histidine uptake system and is concentrated within the cell. Efficacy of the drug is thereby increased 10-fold and at concentrations of 10^{-5}M will completely denude the cells of decarboxylase activity and histamine. Isotopic studies have indicated rapid equilibrium of histidine (within 5 min) between the medium and the cytosol histidine pool, conversion to histamine in the cell cytosol and subsequent translocation of histamine into the granular storage pool. Like immature mast cells, 2H3 cells store very little histamine, leak substantial amounts into the medium and contain small amounts of sulfated polysaccarides in the granules.

Histidine uptake and decarboxylation show dramatic changes during cell growth. These functions develop rapidly in young cells and in 2H3 cells, and reach maximum activity as the cells approach the "S" phase. They then decline to very low activity as cells approach division. These changes can be attributed in part to changes in the number of membrane histidine transport carrier sites. In

differentiating mast cells, histamine synthetic activity declines as cells reach maturity and histamine levels reach their maximum. Although IgE receptors are present in adequate numbers to stimulate degranulation in young cells, the ability to release histamine develops only as mast cells mature or as 2H3 cells proceed through the G_1 phase. We have developed the ability to isolate nonsecretory from secretory cells and this has led us to study degranulation mechanisms in these different cell populations.

Studies were initiated by Dr. Beaven at the Univ. of Cambridge to use the Ca^{2+} fluorescent probe, quin 2, to measure changes in cytosol calcium concentrations ($[Ca_i]$) and to use the assay techniques established by Michael Berridge to measure phosphatidyl inositol (PI) turnover in 2H3 cells. These studies have now clearly established that an increase in $[Ca_i]$ was an obligatory signal for histamine release. defined the quantitative relationships between the Ca signal and histamine release, and shown that the Ca signal was a dynamic response in which antigen stimulation resulted in at least a 10-fold enhancement of Ca^{2+} influx from the medium through specific La^{3+} inhibitable channels. The rate of Ca^{2+} efflux was also increased by the increase in $[Ca_i]$. A second Ca^{2+} independent process which led to progressive inactivation of the stimulatory events was also initiated upon antigen addition. Substantial (up to 70%) breakdown of PI and related phosphoinositides was associated with the stimulated Ca^{2+} influx. PI breakdown occurred only in the presence of external Ca^{2+} but it was not a consequence of Ca^{2+} influx as indicated by absence of PI breakdown upon Ca^{2+} repletion of Ca^{2+} depleted 2H3 cells or upon addition of Ca^{2+} ionophores. Although the data were consistent with PI breakdown being a component of the mechanism by which Ca^{2+} influx was increased, the possibility that stimulation of PI breakdown and Ca^{2+} influx were independent events triggered by IgE receptor activation could not be ruled out. However, our recent findings that nonhistamine releasing clones of the 2H3 cells show no PI or Ca^{2+} responses to antigen stimulation or that PI and Ca^{2+} responses were uncoupled from histamine release during cell mitosis open promising lines of investigation in this area.

2) Sites of histamine inactivation: Studies with vascular endothelial cells. In man and other species, large number of mast cells are present in the adventitia blood vessels. Infusion of histamine in dogs indicate 70-99% clearance of histamine upon single passage across most vascular beds. From these and other data we suspected that the vascular endothelial cell was a primary site of histamine inactivation and we have indeed found that these cells from a variety of tissues contain one or both of the two histamine inactivating enzyme activities (diamine oxidase and histamine methyl transferase). We have now shown that histamine methyl transferase activity is released to the external environment and that extracellular histamine is methylated in suspensions of intact endothelial cells. Further external binding sites with high affinity for diamine oxdiase (DAO) have been characterized. Bound DAO activity, like lipoprotein lipase activity, is readily displaced from endothelial cells by heparin at concentrations of 5 units/ml or lower. The kinetics of binding were consistent with separate binding sites for both enzyme activities but with some overlap in binding with higher concentrations of either enzyme. The binding capacity of the cells was sufficient to yield tissue-bound DAO activity as high as that found in placenta.

3) Pathways of Inflammation. Inflammatory reactions provoked by injection of inflammatory stimulants into the pleural cavity of rats can be divided into two phases. The first mediated by histamine or arachidonate metabolites is the effusion of plasma proteins. This phase is of rapid onset following mast cell degranulation or of slow onset as in the response to carrageenan. The response to carrageenan appears to involve interaction of polysaccharide with pleural cells (probably macrophages) to release arachidonate metabolites. Studies with isolated pleural cells prelabeled with ^{14}C-arachidonic acid indicate that various concentrations of carrageenan cause the release of ^{14}C-label at a rate that is consistent with earlier observations in vivo. The second phase of the inflammatory response is a slow progressive accumulation of neutrophils in the pleural cavity. We have indirect evidence that neutrophil chemotactic activity can be released from mast cells in response to anti-IgE antibody or can be generated by activation of plasma complement components in response to carrageenan injection. The chemotactic factor generated by carrageenan is of high molecular weight, is destroyed by proteolytic enzymes and is partially neutralized by a C5a antibody. Guinea pigs deficient in components of the 'classical' complement activation sequence show that the normal response to carrageenan and chemotactic activity is probably generated through the 'alternate pathway' sequence. Activation of the complement system to generate chemotactic factor(s) in the carrageenan model may thus be a consequence of initial plasma exudation in response to release of arachidonate metabolites from pleural cells. The entire sequence can be blocked by administration of inhibitors of arachidonate metabolism.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00617-08 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The mechanism of carrageenan induced inflammation in rat

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Theresa N. Lo	Research Chemist	LCP	NHLBI
Others:			
Michael A. Beaven	Deputy Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.6	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chemotactic activity for neutrophils was demonstrated in exudates collected at 1 h as well as 4h after the intrapleural injection of carrageenan. The chemotactic activity per mg of 1h exudate protein was much greater than that of the 4h exudate. However, the activity of either exudate was inhibited by monospecific antisera to complement component 5 and was destroyed by trypsin and chymotrypsin treatment. Indomethacin (5 mg/kg, i.v.) reduced equally protein content (56% for the 4h exudate and 69% for the 1h exudate) and total chemotactic activity (58% for the 4h exudate and 62% for the 1h exudate): i.e., chemotactic activity per mg exudate protein was unchanged for these two different aged exudates. These and other data suggest that the exudate chemotactic activity is generated from plasma protein and that indomethacin acts primarily to reduce extravasation of plasma and consequently generation of chemotactic activity. Cells isolated from the pleural cavity (largely macrophages, mast cells, eosinophils and lymphocytes) and incubated with radiolabeled arachidonic acid released substantial amounts of radiolabel upon exposure to carrageenan (1-100 µg/ml) or antisera to IgE. The time-course and concentration-dependency of this release was compatible with the in vivo actions of these two agents.

304

Project Description:

Objectives: In past years, our research activity has been mainly confined in the whole animal in which a given inflammatory agent is injected into the pleural cavity of the rat. Briefly, we have demonstrated that, arachidonate metabolites of non-mast cell origin mediate the carrageenan induced inflammation; histamine is the predominant mediator of the inflammatory response to anti-IgE and compound 48/80; C3a, histamine, and perhaps arachidonate metabolites of mast cell origin participate in the inflammatory response to dextran SO_4 injection; and fluid accumulation following intrapleural injection of 5-60 mg dextran T10 is probably a consequence of osmotic effects. The main target cells with which the inflammatory agent may interact include: 1) loosely attached pleural cells which consist of macrophages, lymphocytes, eosinophils and mast cells (these cells will be referred to as mixed pleural cells hereafter); 2) the visceral and parietal pleural cells; and 3) the cells of subpleural loose connective tissues. A plausible inference is that the inflammatory agent (except dextran T10) exerts its effect by interacting with specific cell population(s) within the pleural cavity, thereby causing the release of distinct inflammatory mediator(s). This year we have initiated studies with isolated pleural cells to identify the primary mediators that lead to the inflammatory responses in vivo. Preliminary data have shown that carrageenan and anti-IgE indeed cause the release of arachidonate metabolites from the isolated mixed pleural cells in vitro. We also include data which lend credence to the hypothesis that the neutrophil chemoattractant generated upon the intrapleural injection of carrageenan is derived exclusively from plasma protein.

Methods Employed: Injection of agents and processing of exudate. Carrageenan (0.5% w/v), dextran T10 (6% to 30% w/v) and compound 48/80 (0.5% w/v) were dissolved in normal saline. The ionophore A23187 (10 mM) was first dissolved in dimethyl sulfoxide and diluted to appropriate concentration in Hank's-HEPES-BSA immediately prior to use. Indomethacin (2.5 mg/ml) was dissolved in dimethyl sulfoxide, diluted with normal saline, and then neutralized with NaOH. Lyophilized antiserum to IgE (Miles Laboratory) was reconstituted in water and serial dilutions were made in normal saline. Rats were given vehicle or indomethacin (5 mg/kg) intravenously. Thirty min later they were lightly anesthetized with ether. Carrageenan (0.1 ml) was injected into the pleural cavity. One hour later, rats were killed by ether, the exudate was aspirated into a plastic syringe, and the cavity was washed with 1 ml of the Gey's medium. Total and differential cell counts were determined by standard techniques described in our previous publications. The remainder of the exudate was centrifuged at 600 x g for 10 min at 4°C. Samples of the supernatant fluid were assayed for protein by the method of Lowry and for chemotactic activity as described in detail in last year's report (Z01 HL 0617-7 LCP).

Treatment of the cell free fraction of exudate with insolubilized trypsin and α-chymotrypsin bound to agarose: Samples (15 mg protein), diluted with Gey's medium, were incubated (37°C) with 25 BAEE units of insoluble trypsin (attached to cross-linked beaded Agarose) and 12.5 ATEE units of α-chymotrypsin (attached to carboxymethyl cellulose) in a total volume of 3 ml for 10 min and 30 min. The reaction was terminated by centrifugation (100 x g for 5 min, 4°C). The supernatant fraction was assayed for chemotactic activity. Controls were treated simi-

larly except that insoluble trypsin and α-chymotrypsin were replaced by the same amounts of cross-linked Agarose and carboxymethyl cellulose.

Isolation of mixed pleural cells: Labeling with [^{14}C]-arachidonic acid. Male Sprague Dawley rats (250-280 g) were killed in ether and the chest was opened by making an incision below the diaphragm and cutting open along both sides of the rib cage. Care was taken not to rupture large blood vessels. The cavity was washed with 2x6 ml Hank's medium and the washes were centrifuged at 850 x g for 10 min at 4°C. The pellet was resuspended in 30 ml of a Hank's -25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) medium and centrifuged (850 x g for 10 min, 4°C). The pellet was suspended in Hank's 25 mM HEPES containing 0.1% fatty acid free bovine serum albumin (Hank's-HEPES-BSA) to give a concentration of 1.5 x 10^6 cells/ml. An aliquot of [^{14}C]-arachidonic acid ([^{14}C] ARA, 56.9 mCi/mmol, NEN, MA) was added to the cell suspension to give approximately 150,000 dpm/ml. Incorporation of [^{14}C] ARA was allowed to proceed for 90 min, 4h or 17h at 37°C. After incubation, free [^{14}C]ARA was removed from radiolabeled cells by washing twice with 30 ml Hank's-HEPES-BSA. The final cell pellets were resuspended in Hank's-HEPES-BSA to give 1 x 10^6 cells/ml, and 0.36 ml aliquots were pipeted into Eppendorf tubes for study of release of radiolabel.

Measurement of radioactivity released from mixed pleural cells. In all release studies, 40 µl of the stimulant solution previously equilibrated to 37°C was added to 0.36 ml cell suspension at 37°C. At timed intervals, the reaction tube was centrifuged (18,000 xg) in the cold room for 90 sec. A portion of the supernatant fluid was withdrawn and assayed for radioactivity in a liquid scintillation spectrometer (Searle, Mark III). Preliminary studies showed that labeled mixed pleural cells continuously released small amounts of radioactivity in the absence of stimulants. All values were corrected for this spontaneous release. Total [^{14}C]ARA incorporated into the cells was determined by treatment of cells with 0.1% Triton X-100 for 1 h at 37°C and assay of the cell lysate for radiolabel. From the known total cellular radioactivity, the results of release experiments are expressed as average percentage of ^{14}C released from cells from triplicate determinations.

Major Findings: Further evidence for the protein nature of exudate chemotactic activity. In last year's report, we tentatively identified the chemoattractant(s) for neutrophils to be C5a-like. We now present data to substantiate the protein nature of the chemoattractant(s). Incubation of the extracellular fraction of exudate with insolubilized trypsin and α-chymotrypsin for 10 min and 30 min at 37°C resulted in, respectively 81 + 4% and 84 + 1% loss of chemotactic activity. No loss of the activity was observed when the sample was incubated with cross-linked beaded Agarose or carboxymethyl cellulose alone.

Effect of indomethacin treatment on generation of chemotactic activity 1h or 4h after intrapleural injection of carrageenan. Appreciable chemotactic activity was present in exudates collected at 1h as well as 4h and this activity was proportional to the amount of exudate protein.

In terms of chemotactic activity per mg of exudate protein, however, values at 1h were greater than those at 4h (ΔLI for 1h exudate is 30 + µm/mg and for 4h exudate is 8.4 ± 1.3 µm/mg). As previously observed with the 4h exudate

indomethacin treatment (5 mg/kg i.v. 30 min before injection of carrageenan) resulted in approximately similar reductions in volume, cell number, protein content and chemotactic activity of exudates collected at 1 hr after carrageenan injection. There was also no evidence that the chemotactic activity per mg of exudate protein from control and indomethacin-treated groups of rats was different. Thus while the chemotactic activity per mg protein was unchanged between control and indomethacin treated rats, total chemotactic activity generated was reduced in proportion to exudate protein content. These findings further support the conclusion that the precursor of chemoattractant(s) for neutrophils is derived from plasma protein.

Incorporation of [^{14}C] arachidonic acid into mixed pleural cells as a function of time. The amount of radioactivity incorporated into the cells was highest after 4 hr incubation (20722 + 454 dpm), intermediate after 90 min incubation (17323 + 625 dpm) and lowest after 17h incubation of cells with [^{14}C] ARA (3948 + 177 dpm). The label was incorporated into the cellular phospholipid pool as demonstrated by a variety of extraction and precipitation techniques. It was not removed by extensive washing of cells but as reported in Methods, there was slow spontaneous release of label over 60 min at 37°.

Stimulation of radioactivity released from mixed pleural cells by carrageenan and anti-IgE. As little as 1 µg/ml (final concentration) of carrageenan stimulated significant release of radioactivity. Release was dependent on the concentration of carrageenan over the range of 1-25 µg/ml. The maximum response (20% release of cellular label was observed with 25-100 µg of carrageenan). Similarly, a concentration dependent release of radioactivity was observed with anti-IgE: for example 3%, 5% and 6% of label was released from mixed pleural cells upon incubation with 1:80, 1:40, and 1:10 dilution of antiserum to rat IgE, respectively. The time course of release was similar to that observed with carrageenan.

Specificity of response in vitro. The action of carrageenan was consistently observed with the mixed pleural cells. Preliminary studies also indicated that adherant pleural cells (to plastic petri dishes) responded better to carrageenan (up to 20% release) than do nonadherant cells. Carrageenan (100 µg/ml) did not stimulate the release of radioactivity from rat basophilic leukemia cell cultures or cultured rat connective tissue fibroblasts. The fibroblast cultures did, however, respond to 0.2 µg/ml of the calcium ionophore A23187 (8% release of label).

Significance to Biomedical Research and the Program of the Institute. Although there is no consensus as to the identity of substance(s) mediating neutrophil infiltration in response to carrageenan, the bias of many authors is toward the arachidonate metabolites to the exclusion of other potential mediators. Our data suggest that plasma complement may have an important role in this response but that arachidonate metabolites are responsible for changes in vascular permeability as an early event in the inflammatory response. The in vitro studies will complement the findings of our previous in vivo studies and further our understanding of the mechanism(s) underlying various types of inflammation.

Proposed Course of Project: Studies will be conducted with purified pleural cell populations to identify the specific cell type responding to carrageenan and other selected agents. A variety of separation techniques established in the laboratory will be employed to isolate mast cells, macrophages, and lymphocytes. HPLC procedures will be used to identify and quantitate radiolabeled cellular phospholipids and released compounds.

Publication:

Lo, T.N., Almedia, A.P. and Beaven, M.A.: Effect of indomethacin on generation of chemotactic activity in inflammatory exudates induced by carrageenan. European J. Pharmacol. 99: 31-43, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00620-07 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of histamine synthesis and release in tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Elizabeth WoldeMussie	Staff Fellow	LCP	NHLBI
Others:			
Michael A. Beaven	Deputy Chief	LCP	NHLBI
Diane Aiken	Biologist	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	0.9	0.7

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Uptake of labeled histidine into rat basophilic leukemia (2H3) cells was time and temperature dependent. This uptake system had high affinity for histidine (K_m, 24 ± 4 μM). The histidine was decarboxylated to form histamine and this histamine was incorporated to storage granules. However, some of it was lost to the medium. In elutriated cells histidine uptake and decarboxylation were low in small cells and progressively increased with cell size, but decreased as the cells approached cell division. The changes in kinetic constants suggested a decrease in number of active sites for histidine transport as cells approach cell division. α-fluoromethyl histamine (α-FMH), a suicide inhibitor of histidine decarboxylase, like histidine was taken up by 2H3 cells but had lower affinity (Km, 170μM) for the uptake system than histidine. α-FMH partially inhibited the uptake and decarboxylation of histamine. In cultured cells (1-4 days) low concentrations of αFMH stimulated histidine decarboxylase activity while high concentrations were inhibitory. Histamine levels changed accordingly. However αFMH had no effect on cell division.

Project Description:

Objectives: Rat basophilic leukemia (2H3) cells like mast cells have high histamine synthetic activity. Our objective was to study the uptake of histidine and synthesis and storage of histamine throughout the growth cycle of the cell. We also studied the effect of inhibition of histamine synthesis by α-fluoromethylhistidine (α-FMH) on the growth and development of these cells.

Methods Employed: The 2H3 cells in culture were maintained and subcultured in supplemented minimum essential medium (MEM) as previously described (Z01 HL 00620-06). Cells in confluent cultures were separated into fractions of different sizes by elutriation (J. Pharm.Exptl.Therap.224:620,1983).

Assay procedures. Histamine content of samples were determined by enzymatic isotopic assay (J. Pharmacol.Exp.Ther.224:620,1983). DNA was determined by a microfluorescence procedure (J. Pharmacol.Exp.Ther.223:440,1982). To measure histamine synthetic activity, cells were incubated with ^{14}C(carboxyl) histidine and the rate of $^{14}CO_2$ released was measured (Biochem.Pharmacol. 31:1189,1982).

Histidine uptake: a) Time-course studies - Cells, cultured in cluster plates overnight, were incubated with ^{14}C(ring labeled) histidine alone or with 250 μM unlabeled histidine for various periods at 37° or 4°C. The medium was quickly removed and the cells were washed and lysed with 200 μl H_2O. Radioactivity was determined in 100 μl aliquots. b) Kinetics studies - Cells were incubated with ^{14}C(ring) histidine and various concentrations of unlabeled histidine for 1 min. They were washed and lysed as above and radioactivity determined. The rate and kinetics of αFMH uptake were determined by similar methods. Further characterization of uptake of histidine or αFMH was determined by methods described previously (Z01 HL 00620-06 LCP).

Major Findings: Histidine uptake. The uptake of labeled histidine into 2H3 cells was both time and temperature dependent. At 37°C uptake occurred initially at a rapid rate followed by a slower rate of uptake over the period of one to two hours. After 1 hr the concentration of label in the cells was 75 times that in the medium.

The uptake was by two components. With low concentrations uptake occurred through a high affinity (K_m - 24 + 4 μM) system and with high concentrations uptake occurred through a lower affinity system. The lack of saturation of uptake indicated that uptake did not occurr solely by a single transport system.

Histamine synthesis and storage - Conversion of labeled histidine to histamine was apparent within a few minutes (5-10) and continued at a steady rate thereafter. This rate paralleled the slower (2nd) rate of histidine uptake which indicated that histidine was taken up to compensate for that converted to histamine. Some of the newly synthesized histamine was incorporated into storage granules and could be released (33 + 1% versus 49 + 1% release of endogenous amine) by stimulation with antigen from cells previously primed with IgE. There was also a continuous loss of histamine into the incubation medium (equivalent to 25% of that in cells by 3 hr). However, as the amount of histamine in the cells remained constant, we calculated the half-life of histamine in these cells to be about 12 hours.

Histidine uptake and decarboxylation in elutrated fractions of 2H3 cells. Of the 12 fractions collected most of the cells were recovered in fractions 4 to 7. The DNA content per cell was nearly the same in fractions 3 to 7, but increased in fractions 8 to 10 indicating that the cells in fractions 8 to 10 may be in the S phase of the cell cycle. Histamine levels, rates of histidine uptake and decarboxylase activity paralleled each other in the different fractions. They were low in fractions of small cells, highest in fractions of cells approaching S phase and decreased in fractions of cells nearing cell division.

The activity of the high affinity component of histidine uptake described above was low in of small cells (3 and 4), reached maximum in fraction 7 and decreased again in cells from fraction 9. These changes were attributable to change(s) in values for V_{max} and not in K_m. Thus the number of uptake sites were altered during the cell cycle. It is interesting to note that since the histamine level in the cells changed in parallel histidine decarboxylase activity, the cells did not retain histamine for long periods after histamine synthetic activity had decayed.

Uptake of αFMH into 2H3 cells. Labeled αFMN, a selective and irreversible inhibitor of histidine decarboxylase activity, was also taken into the cells but at a slower than that of histidine. Uptake of both compounds appeared to be competitive and were inhibited to the same degree by various amino acids and analogs. Although both appeared to be taken up by the same carrier mediated system, the affinity of the system was lower for αFMH (K_m 170 μM) than for histidine (24 μM).

Inhibition of histidine uptake and histidine decarboxylase activity in 2H3 cells by αFMH. The uptake of histidine into 2H3 cells was inhibited by αFMH in a dose dependent manner but uptake was not completely blocked even with the highest concentration used (10^{-3}M). The inhibition of histidine decarboxylase activity by αFMH, however, was both time and dose dependent and complete inhibition was observed in the presence of 10^{-5}M α-FMH by 60 min.

When 2H3 cells were cultured for 1-4 days in the presence of various concentrations of αFMH, stimulatory and inhibitory effects on histidine decarboxylase activity were noted. At low α-FMH concentrations (<10^{-7}M) histidine decarboxylase activity was increased (up to 134 + 9% of control values) and at higher concentrations decarboxylase activity was inhibited. There was no detectable activity with 10^{-5}M α-FMH after 4 days. Histamine levels paralleled changes in histidine decarboxylase activity. Histamine levels were elevated in cells exposed to 10^{-7}M αFMH and decreased in cells exposed to higher concentrations of drug. Cell growth or division was not affected by αFMH, so these processes do not depend on histamine synthesis.

Significance to Biomedical Research and the Program of the Institute: Even though it is known that histamine is stored in mast cell or basophil granules, it is not clear how this histamine is synthesized and accumulated. This study sheds light on how this takes place in terms of histidine uptake and rate of

histamine synthesis during the cell cycle. Uptake is a relatively rapid process compared to decarboxylation and translocation of histamine into the granular stores. However, in the transformed 2H3 cell as in immature mast cells, storage of histamine is an inefficient process in that considerable amounts of histamine leak into the extracellular environment. Spontaneous eruptions and itching associated with mast cell tumors and tissues with rapidly expanding populations of mast cells (e.g. keloids) might well be due to histamine leakage. The studies with αFMH point to possible use of this drug in arresting histamine synthesis in these conditions, but our findings suggest also that the drug would not arrest tumor growth or mast cell proliferation.

Proposed Course of Project: Further studies will focus on regulation of synthesis and turnover of histidine decarboxylase during mast cell maturation and growth cycle of 2H3 cells. Approaches used will include studies of the mechanism by which low concentrations of αFMH induce decarboxylase activity, use of fluorescent antibodies to quantitate and locate histidine decarboxylase in the cell and the effect of drugs that inhibit protein synthesis or arrest cell cycle on enzyme activity. The program will complement studies of mechanism of mast cell degranulation as reported in project Z01 HL 00620-06 LCP.

Publications:

Beaven, M.A. and WoldeMussie, E.: Histamine in body fluids: Its measurement in different clinical states. New England and Regional Allergy Proceedings, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00805-02 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Anti-P-450 monoclonal antibodies: Effect on drug metabolism in different tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Henry A. Sasame	Chemist	LCP	NHLBI
Other Investigator James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Harry V. Gelboin, Laboratory of Molecular Carcinogenesis, NCI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NIH, NHLBI-IR-LCP, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A monoclonal antibody (anti 3MC 1-7-1) raised against a cytochrome P-450 induced by 3-methylcholanthrene in rats, blocked the conversion of R and S propranolol to 4-hydroxypropranolol (4OHPr), 5-hydroxypropranolol (5OHPr), and desisopropylpropranolol (DIP) by microsomes from the small intestine of rats treated with β-naphthoflavone (βNF). The ratios of the products formed from R-propranolol, namely 4OHPr/5OHPr and DIP/5OHPr, by the antibody sensitive portions of the enzymatic activities in the microsomes of liver, kidney, lung and small intestine of βNF-treated rats were remarkably similar. In addition, this monoclonal antibody blocked effectively the conversion of R-Pr to 4OHPr, 5OHPr and DIP in microsomes from stomach and colon of rats.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: Since propranolol is usually administered orally it is of interest to investigate the extent to which propranolol can be metabolized by enzymes in the GI tract. Previously we have shown that treatment of rats with β-naphthoflavone (βNF) enhanced greatly the rate of propranolol metabolism by liver microsomes, whereas treatment with phenobarbital had little effect. Using a monoclonal antibody, we have extended our study to elucidate the mechanism of propranolol metabolism by GI microsomes.

Methods Employed: The formation of 4OHPr and DIP, which is one major metabolites of R-propranolol was assayed in a high performance liquid chromatography equipped with a fluorescence detector [FS970 LS fluorometer, Schoeffal Instrument Corp.)

Major Findings: 1) The monoclonal antibody blocked the metabolism of both R and S propranolol by intestinal microsomes from untreated as well as BNF treated rats, whereas in liver this antibody blocked only enzymes in microsomes from BNF treated rats.

2) The cytochrome P-450 blocked by the monoclonal antibody in microsomes from small intestine catalyzed the formation of the metabolites of R and S propranolol at different rates. The R/S ratios for 4OHPr and DIP were 1.59 and 2.19, respectively. On the other hand, there was no stereospecificity toward the formation of 5OHPr from the two enantiomers.

3) The administration of sesame oil, a commonly used vehicle for water immiscible chemicals, increased the rate of R-propranolol metabolism 2 to 3 fold in small intestine but to a smaller extent in liver. Activities in both organs were blocked by the monoclonal antibody.

4) The monoclonal antibody blocked R-propranolol metabolism by microsomes in stomach as well as those in colon isolated from BNF treated rats. However, the relative rates of propranolol metabolism by microsomes in small intestine, colon, stomach were 10, 5, 1, respectively. The metabolic profile in colon microsomes in small intestine were very similar with respect to their stereospecificity and regioselectivity. The profile in stomach could not be estimated due to very low enzymatic activity.

Significance to Biomedical Research and to Program of the Institute: The development of a monoclonal antibody that only recognizes a single P-450 in microsomes would truely shed light on the mechanism of *in vitro* propranolol metabolism in various tissues.

Proposed Course of Project: It would be important to evaluate the effect of different inducers on drug metabolism by GI tract, which may lead to a development of therapeutic drugs against carcinogenesis.

Publciations: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00937-02 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *80 characters or less. Title must fit on one line between the borders.)*

Biochemical mechanisms of mast cell degranulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Michael A. Beaven Deputy Chief LCP NHLBI

Others:

Elizabeth WoldeMussie Staff Fellow LCP NHLBI

COOPERATING UNITS *(if any)*

Dr. Reuben Siraganian, NIDD, Lab. Microbiology and Immunol.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.9	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Increases in free cytoplasmic calcium (Ca^{2+}) levels as measured by the fluorescent Ca^{2+} indicator Quin-2, release of inositol phosphates from phosphatidyl inositol and histamine release were examined in histamine-releasing (2H3) and nonreleasing (1A3) clones of rat basophilic leukemia (RBL) cells. In cells passively sensitized with monoclonal IgE, cross-linking of IgE with antigen or concanavalin-A resulted in release of inositol phosphates (PI response), increase in cytosol Ca^{2+} levels (Ca response) and histamine release in 2H3 cells. None of these responses were observed in 1A3 cells. In 2H3 cells, cytosol Ca^{2+} reached maximal level (1 μM) by 2.75 min and then declined over the course of 40 min to levels observed before stimulation (0.11 μM). Rates of inositol phosphate release were correlated with the actual increase in cytosol Ca^{2+} level (i.e.$\Delta[Cai]$) whereas a correlation between histamine release and $\Delta[Ca_i]$ was observed only when $[Ca_i]$ had reached maximum levels. All 3 responses were dependent on continued presence of 3 factors; external Ca^{2+}, ligand and cellular ATP production and showed similar concentration-response relationships to varying concentrations of ligand or external Ca^{2+}. Removal or blockade of any of the factors resulted in arrest (within 40 sec.) of all responses. Although histamine release was dependent on increases in cytosol Ca^{2+}, it could be uncoupled from the two other events. Histamine release, but not PI or Ca responses, was suppressed in cells arrested in mitosis with Nocodazole or in cells exposed to 100 μM Zn^{2+}. PI response could not be uncoupled from the Ca response. Although the above results indicate that both PI and Ca response are intimately associated, the data provide no clear indication of the relationship between the two.

3,5

Project Description:

Objectives: Release of histamine and other inflammatory mediators from tissue mast cells and blood basophils is the primary event in a variety of acute allergic reactions. Release of granules from these cells is an energy and calcium-dependent process which is initiated by cross linking of membrane bound IgE molecules by antigen or other cross linking agents. Stimulated biochemical changes are similar to those observed in other secretory cell systems, namely, enhanced phospholipid metabolism, release of arachidonic acid, cyclic AMP production and activation of protein kinases. However, the importance (or existence) of some of these changes is controversial and their relationships are unclear. A key event appears to be either influx of Ca2+ from external sources or mobilization of Ca2+ from the intracellular pool but assumed increases in cytosol Ca2+ levels ($[Ca_i]$) have not been previously measured.

Last year the principal investigator initiated studies in Dr. Metcalfe's laboratory in Cambridge, England to observe and quantitate changes in $[Ca_i]$ directly by use of the fluorescent probe, quin 2. For convenience, studies were conducted in a histamine releasing clone (2H3) of cells derived from the rat basophilic leukemia (RBL) cell line. Histamine release was observed to be directly dependent on increases in $[Ca_i]$ and was accompanied by extensive breakdown of phosphatidyl inositol in the plasma membrane to form inositol phosphate(s) (see last year's report). This year we have attempted to assess whether or not PI breakdown is the mechanism by which the Ca signal is generated and maintained. The present studies involved more sophisticated analysis than previous studies of the 3 known inositol phosphates, inositol (1)-Mono-,(1,4)-bis-,and (1,4,5)-trisphosphate. These products are derived respectively from phosphatidyl inositol, phosphatidyl inositol 4-phosphate and phosphatidyl inositol 4,5-bisphosphate by the action of one or more PI phosphodiesterases. A current hypothesis is that inositol (1,4,5) trisphosphate formed by hydrolysis of PI (4,5) bisphosphate is the compound involved in recruitment of Ca^{2+} ions from cell membranes (Streb et al., Nature 306:67,1983) and that the parent PI bisphosphate is formed by sequential phosphorylation of PI (i.e. PI -> PI (4) monophosphate -> PI (4,5) bisphosphate) by the action of specific kinases.

Methods Employed: 1) Cell lines (1A3 and 2H3) were supplied by Dr. Reuben Siraganian. These were cultured and prepared as described in last years report except that cultures of the 1A3 line contained Bromodeoxyuridine, 10^{-4}M. Rat mast cells were isolated and purifed by elutriation Beaven et al. (J.Pharm.Exptl. Therap. 224:620, 1983).

2) Experimental procedures: Techniques for measurement of $[Ca_i]$ in suspensions of quin-2 loaded cells and measurement of PI breakdown was described in detail in last years report. Studies of PI breakdown were performed in cluster plate cultures or suspensions of quin-2 loaded cells. The cellular PI pool was labeled by overnight incubation of cultures with ^3H-labeled inositol. Cells were passively sensitized with a monoclonal IgE antibody specific for ovalbumin as previously described. Degranulation was induced by addition of aggregated ovalbumin or other stimulants as indicated. Histamine release was determined by radioenzymatic assay (J. Pharm.Exptl.Therap. 224:620, 1983) and release of total water soluble inositol phosphates by absorption onto Dowex formate columns and elution

with a sodium formate buffer. Separation of individual inositol phosphates was achieved by sequential elution from the Dowex formate columns with ammonium formate buffers of different ionic strengths according to the procedures of Berridge (Biochem. J. 212:849,1983). Residual PI was extracted into chloroform and, after washing the chloroform extract to remove water soluble labeled material, assayed by liquid scintillation spectrometry.

Major Findings: Time course of histamine release, changes in $[Ca_i]$ and PI breakdown in 2H3 cells. The characteristics of the various responses described in last years report (from Cambridge) have been reproduced in experiments by myself and coworkers at the NIH. That is addition of antigen (cross-linked ovalbumin) to quin-2 loaded 2H3 cell suspension produced an increase in calcium-dependent fluorescence. $[Ca_i]$ increased from 0.11 to over 1.0 μM and reached a maximum by 2.75 min. Thereafter $[Ca_i]$ slowly declined over the course of 40 min to levels observed before addition of stimulants. Histamine release was apparent within 30-60 sec but for technical reasons it was uncertain if the increase in $[Ca_i]$ preceded histamine release. However, curve fitting of all data by computer analysis indicated a correlation between $\Delta[Ca_i]$ and rates of histamine release after $[Ca_i]$ had reached a maximum (i.e. after 2.75 min). PI breakdown was observed within 10-40 sec of addition of antigen and at all times thereafter rates of inositol phosphate release were correlated with $\Delta[Ca_i]$. Although in some experiments the appearance of inositol phosphate was coincident with the first detectable increase in $[Ca_i]$ there has been no indication that PI breakdown preceded $[Ca_i]$ increase.

We reported last year that all three responses; $[Ca_i]$ increase, histamine release and PI breakdown were highly correlated (R 0.99, $p < 0.01$) with antigen concentration and similar significant correlations have now been observed between the different responses and varying concentrations of external Ca^{2+} $[Ca_o]$. With low $[Ca_o]$ (i.e. < 50 μM) or presence of the Ca^{2+} channel blocker, La^{3+}, all 3 responses were blocked but could be initiated by addition of excess (1 mM) $[Ca_o]$ or Ca^{2+}/EGTA to chelate La^{3+} ions. In addition to the requirement for external Ca^{2+} ions, all responses were dependent on intracellular supply of ATP. Once ATP levels had been reduced to less than 200 μM with sodium azide and 2-deoxyglucose the cells were completely refractory to antigen stimulation.

After the responses had been initiated by antigen the addition of excess EGTA (1.1 mM), La^{3+} (10 μM) or metabolic inhibitors resulted in rapid decline in $[Ca_i]$ to prestimulated levels within 30 sec. Histamine release and PI breakdown also ceased. In nonstimulated cells the same reagents produced little or no perturbations in $[Ca_i]$.

As indicated by the above the calcium response or signal was dependent on continued presence of ligand, $[Ca_o]$ and cellular ATP and operated through the appearance of La^{3+} sensitive channels which are absent in nonstimulated cells. Further the calcium signal is not due to a single influx of Ca^{2+} ions but is maintained by a dynamic balance between enhanced Ca^{2+} influx and efflux. Interferance in Ca^{2+} influx (with La^{3+}, lowering of $[Ca_o]$, or removal of stimulant) leads to rapid decline in $[Ca_i]$ presumably through an ATP-dependent Ca^{2+} pump which continues to operate at low ATP levels (i.e. > 200 μM). It may be noted that an ATP-depen-

dent Ca^{2+} pump with a low apparent K_m for ATP (4μM) has been observed in erythrocyte and lymphocyte plasma membrane (Lichtman et al., J. Biol. Chem. 256:6148,1981).

2) Uncoupling of histamine release from the Ca signal. Last year we reported that histamine release, but not the Ca response and PI breakdown, were inhibited by Zn^{2+} at concentrations of 50μM. Partial inhibition of release was observed with concentrations of 10 μM. We have since shown that histamine release was uncoupled from the Ca response in cells arrested in miotisis by the microtubule-disrupting drug Nocodazole (0.04 μg/ml). Treated cells (92% in mitosis) showed the normal increase in $[Ca_i]$ but did not release histamine. Removal of drug by washing resulted in restoration of histamine-release response within 2 hr. The drug itself had no effect on histamine release in interphase cells. Inhibition of histamine secretion was anticipated because of previous findings that certain intracellular processes, fusion of intracellular vesicles, endocytosis and phagocytosis are switched off during mitosis. We have also shown (see project report Z01 HL 00620-07 LCP) that histidine uptake and incorporation of histamine into granular stores is suppressed in 2H3 cells during cell division. The mechanism by which degranulation is selectively suppressed remains to be determined but the mechanism does have an important bearing on the overall process by which transduction of stimulatory signal to secretory events are coupled.

3. Detailed analysis of the PI breakdown in 2H3 cells. PI breakdown was always correlated with changes in $[Ca_i]$ when cells were stimulated with IgE cross linking ligands but negligible breakdown was observed upon stimulation with the ionophore A23187 or addition of Ca^{2+} to Ca^{2+} depleted cells (see last year's report). Thus PI breakdown was not a consequence of Ca^{2+} transport into the cell nor was it required for histamine release. Also as discussed above PI breakdown was not, as it is in other cell systems, a Ca^{2+}-independent event.

We have now shown that inositol (1,4)-bisphosphate is the first major breakdown product to appear after antigen addition. By 5-10 min inositol (1)-monophosphate is the predominant metabolite. At all times inositol (1,4,5)-trisphosphate remains a minor (< 10% of total inositol phosphates released) product of PI breakdown. The sequence of hydrolysis of the various phosphoinositides and importance of the inositol trisphosphate in the generation of the calcium signal is, therefore, unclear at this time.

4) Studies in 1A3 cultures. These cultures showed no change in $[Ca_i]$, and negligible release of histamine (<3%) or inositol phosphate (<5% of release in 2H3 cells) upon addition of optimal (10 μg/ml) and supraoptimal (100 μg/ml) concentrations of antigen. This cell line possesses the same ability to bind IgE as do 2H3 cells (Siraganian unpublished data). The 1A3 cells did, however, incorporate labeled inositol into the PI pool to the same extent as did 2H3 cells and the ability to synthesize PI did not appear to be defective.

Attempts to load rat peritoneal mast cells with quin-2 have so far been unsuccessful. Nor have we been able to extensively label the PI pool by incubation with labeled inositol.

Significance to Biomecical Research to the Program of the Institute: The 2H3 cell has proved to be a useful model to study relationships of calcium flux and PI turnover to secretory response. By analogy, the findings have important implication for studies of calcium-dependent secretory responses in other cell types. The present studies indicate that 1) IgE receptor aggregation increases membrane permeability to Ca^{2+} ions by an ATP-dependent process and as a consequence $[Ca_i]$; 2)increases in cytosol $[Ca_i]$ are directly correlated with histamine release but the two events can be uncoupled with Zn^{2+} or mitotic arrest; 3) the calcium signal is a dynamic response maintained by at least a 10-fold enhancement in rates of Ca^{2+} influx and this is balanced by enhanced Ca^{2+} efflux; 4) substantial PI breakdown occurs in parallel with changes in Ca^{2+} flux but we are uncertain as to whether or not the two are causal or independent events; 5) as indicated by studies with 1A3 cells further studies with RBL cell clones may provide useful information related to item 4.

Proposed Course of Project: Studies will continue with clones of RBL cells to further define the relationship between the calcium signal and PI response. Preliminary experiments with vascular endothelial cells and neutrophils have indicated that the cells can be loaded with quin-2 and changes in $[Ca_i]$ assessed. The same approaches will be utilized to study mechanisms related to calcium-coupled responses in these cell types.

Publications:

Rogers, J., Hesketh, T.R., Smith, G.A., Beaven, M.A.,Metcalfe, J.C., Johnson,P., and Garland, P.B.: Intracellular pH and free calcium changes in single cells using quene-1 and quin-2 probes and fluorescence microscopy. FEBS Letts. 161: 21-27, 1983.

Beaven, M.A., Rogers, J., Moore, J.P., Hesketh, T.R., Smith, G.A. and Metcalfe, J.C.: The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. J. Biol. Chem. 259: 7129-7136, 1984.

Beaven, M.A., Moore, J.P., Smith, G.A., Hesketh, T.R. and Metcalfe, J.C.: The calcium signal and phosphatidyl inositol breakdown in 2H3 cells. J. Biol. Chem. 259: 7137-7142, 1984.

Soll, A.H., Amirian, D.A., Thomas, L.P., Park, J., Beaven, M.A. and Yamada, T.: Gastrin receptors on nonparietal cells isolated from canine fundic mucosa. Am. J. Physiol, in press.

Hesketh, T.R., Beaven, M.A., Rogers, J., Burke, B. and Warren, G.B.: Stimulated release of histamine by a rat mast cell line is inhibited during mitosis. J. Cell Biology 98: 2250-2254, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

[illegible] HUMAN RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00950-03 LCP

October 1, 1983 through September 30, 1984

Diffusion of reactive metabolites into blood

PERSONNEL (Show the Principal Investigator first. Name, title, laboratory, and institute)

P.I. Serrine S. Lau	Visiting Fellow	LCP	NHLBI
Other Investigators:			
Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously reported that the postulated hepatotoxic metabolite of bromobenzene, bromobenzene-3,4-oxide, can be detected in venous blood of rats trapping it as the corresponding [35S]-glutathione conjugates. Moreover, bromobenzene-3,4-oxide is detected in blood of rats treated with phenobarbital and diethyl maleate than in venous blood of rats treated with phenobarbital alone. The half-life of bromobenzene-3,4-oxide in blood is about 13.5 sec and it may therefore contribute to the extrahepatic covalent binding and toxicity observed after bromobenzene administration.

320

Project Description:

Objectives: We have previously developed a method which enabled us to detect the presence of bromobenzene-3,4-oxide in venous blood of rats. We therefore needed to estimate the half-life of bromobenzene-3,4-oxide in blood in order to determine the extent to which this metabolite may contribute to the extrahepatic covalent binding and toxicity observed after bromobenzene administration.

Methods Employed: Methods for the detection of bromobenzene-3,4-oxide in venous blood of rats have been described in a previous report from this Laboratory.

Major Findings: More bromobenzene-3,4-oxide is detected in rats treated with both phenobarbital and diethyl maleate than with phenobarbital alone. Moreover, the half-life of bromobenzene-3,4-oxide in venous blood was estimated to be 13.5 sec by this technique.

Significance to Biomedical Research and Program of the Institute: The results not only confirm our previous findings that reactive metabolites of bromobenzene escape intact hepatocytes and reach venous blood, but also indicate that the 3,4-oxide has a sufficiently long half-life to reach extrahepatic organs. Thus, nearly all of the epoxide leaving the liver (and any other organ in which it is formed) will enter the lung, and the concentration of the epoxide in blood leaving the lung will be nearly identical to its concentration in arterial blood entering the other organs of the body. The extent to which the epoxide in arterial blood contributes to its covalent binding in extrahepatic tissues will depend on the ability of the various tissues to convert the epoxide to inactive metabolites. The technique used in these studies may be used to detect in the blood of experimental animals the presence of reactive intermediates of other compounds and to estimate the stability of these intermediates in blood.

Proposed Course of Project: Terminated

Publications:

Monks, T.J., Lau, S.S. and Gillette, J.R.: Diffusion of reactive metabolites at hepatocytes: Studies with bromobenzene. J. Pharmacol. Exp. Ther. 228: 393-399 1984.

Gillette, J.R., Lau, S.S. and Monks, T.J.: Intra- and extra-cellular formation of metabolites from chemically reactive species. Biochem. Soc. Trans. 12: 4-7, 1984.

Lau, S.S., Monks, T.J., Greene, K.E. and Gillette, J.R.: Detection and half-life of bromobenzene-3,4-oxide in blood. Xenobiotica, in press.

Monks, T.J. and Lau, S.S.: Activation and detoxification of bromobenzene in extrahepatic tissues. Life Sciences, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00953-03 LCP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of kidney necrosis produced by chloroform

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Lance R. Pohl	Chief, Section	LCP	NHLBI
Others:			
John W. George	Chemist	LCP	NHLBI
Hiroko Satoh	Vist. Assoc.	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Pharmacological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It has been known for many years that there are species, strain, and sex differences in the incidence and severity of the nephrotoxicity caused by chloroform. For example, DBA/2J - males are more sensitive than are C57/BL6 male mice and males of a given strain are more sensitive than are females. The molecular basis for these differences, however, has not been clearly understood. In this investigation, we have found that sensitivity to chloroform correlates with the capacity of the kidney to metabolize chloroform to the toxic metabolite phosgene. For instance, kidney homogenates from DBA/2J mice convert chloroform to phosgene approximately twice as rapidly as do those from C57/BL6 male mice, whereas kidney homogenates from ICR males metabolized chloroform to phosgene at nearly an order of magnitude more rapidly than do those from ICR female mice. Cytochrome P-450 in the microsomal and mitochondrial fraction of the kidney appears to catalyze this reaction and testosterone somehow regulates the activity of these enzymes.

322

Project Description:

Objectives: To determine the mechanism of sex and strain differences to chloroform-induced necrosis of renal proximal tubules.

Methods Employed: The metabolism of $CHCl_3$ to phosgene ($COCl_2$) by kidney tissue preparations from DBA/2J, and C57/BL6 male mice and ICR male and female mice were determined by HPLC as described previously. Some of the ICR female mice were administered a single dose of testosterone proprionate (17.9 mg/kg); after three days, their kidneys were removed and used for metabolic studies.

Major Findings: The rate of metabolism of $CHCl_3$ to $COCl_2$ by kidney homogenates was decreased by 95 and 63 percent when NADPH was excluded from the reaction mixtures or when the reactions were conducted in an atmosphere of $CO:O_2$ (8:2). The majority of the enzyme activity appeared to be localized in the microsomal fraction of the cell, although a significant amount of activity was found in the mitochrondria.

Kidney homogenates from male DBA/2J mice metabolized $CHCl_3$ to $COCl_2$ approximately twice as rapidly as did those of male C57BL/6J mice.

Kidney homogenates from ICR male mice metabolized $CHCl_3$ to $COCl_2$ at nearly an order of magnitude more rapidly than did those from female mice. Three days after a single treatment with testosterone proprionate, the metabolic activity of the female mice was increased by over 600 percent. The addition of testosterone to kidney homogenates of untreated female mice had no significant effect on the metabolism of $CHCl_3$ to $COCl_2$.

Significance to Biomedical Research and Program of the Institute: It has been known for many years that the sensitivity of animals to $CHCl_3$-induced nephrotoxicity may vary markedly with the species, strain, and sex of the animals. For example, male DBA/2J mice are more susceptible to this toxicity than are male C57BL/6J mice, whereas the male F_1 hybrids of these two strains are of intermediate sensitivity to $CHCl_3$. In addition, female mice and castrated male mice are resistant to $CHCl_3$-induced nephrotoxicity. Their treatment with testosterone, however, sensitizes them to $CHCl_3$.

In the present investigation, we have found that the biochemical basis for these differences in susceptibility to $CHCl_3$-induced nephrotoxicity is attributed to an enzyme system that metabolizes $CHCl_3$ to the toxic metabolite $COCl_2$. The enzyme appears to be cytochrome P-450 and is found in both the endoplastic reticulum and in the mitochondria fraction of the cell. Interestingly, it appears that testosterone modulates the metabolism of $CHCl_3$ to $COCl_2$ by the kidney. Moreover, since plasma levels of testosterone are 350% higher in DBA male mice than in C57BL male mice, this effect may be responsible, at least in part, for both sex and strain differences in sensitivity to $CHCl_3$-induced nephrotoxicity.

Proposed Course of Project: This project has been completed.

Publications:

Branchflower, R.V., Nunn, D.S., Highet, R.J., Smith, J.H., Hook, J.B. and Pohl, L.R.: Nephrotoxicity of chloroform: metabolism to phosgene by the mouse kidney. Toxicol. Appl. Pharmacol. 72: 159-168 (1984).

Pohl, L.R., George, J.W., and Satoh, H., Strain and sex differences in chloroform-induced nephrotoxicity. Different rates of metabolism of chloroform to phosgene by the mouse kidney. Drug Metabolism Disp. 12: 304-308 (1984).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00956-03 LCP

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathways of inflammatory response in different experimental models in rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Theresa Lo	Research Chemist	LCP	NHLBI
Others:			
Michael A. Beaven	Deputy Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In contrast to the slow accumulation of fluid, plasma proteins and neutrophils observed after intrapleural injection of carrageenan, a second type of response is observed with mast cell degranulating agents, e.g. anti-IgE, compound 48/80, and dextran sulfate. In this response, there is rapid protein and fluid accumulation, which is generally correlated with the extent of histamine release, and at later stages neutrophil accumulation. Fluid and protein but not neutrophil accumualtion is blocked by treatment with antihistamines. The response to dextran T10 fits neither of these two categories. A rapid accumulation of protein- and cell-free transudate is associated with partial mast cell degranulation. This response is not blocked by pretreatment with antihistamines. Further, the extent of fluid accumulation in the pleural cavities of normal and mast cell-deficient mice (WBB_6 F_1/J-W/W^v) is the same. Thus, the response to dextran T10 is probably an osmotic effect rather than a true inflammatory response to mast cell degranulation. The effects of dextran sulfate appeared to be a consequence of both osmosis (fluid accumulation) and response to release of mediators (protein and cell accumulation).

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives: Our previous studies (see report no. Z01 HL 00956-02 LCP) indicated that histamine released from mast cells plays an important role in the anti-IgE or compound 48/80-induced accumulation of fluid and protein. The fluid accumulation in response to the intrapleural injection of dextran T10 was not blocked by pretreatment with antihistaminic drugs and histamine appeared to have no role in this type of response. We have further characterized the dextran response in rats and in the WBB $6F_1$/J-W/W^W mice, a strain of mice in which the total number of mast cells in the skin were less than 1% of the number in the congeneic normal mice +/+ (Kitamura and Hatanaka, Blood 53:447,1978). In addition, a derivative of dextran (i.e. dextran sulfate) that is a known complement activator was also studied to assess the role, if any, of complement in the sequence of events leading to acute inflammation.

Methods Employed: Indomethacin was dissolved in dimethyl sulfoxide, diluted with normal saline and then neutralized with NaOH. Dextran T10 (300 mg/ml; av. MW 10,000), dextran sulfate (10-50 mg/ml; av. MW 500,000), pyrilamine maleate (5 mg/ml) and metiamide (100 mg/ml) were dissolved in normal saline. Drugs were injected subcutaneously 30 min before the intrapleural injection of inflammatory agents (in volume of 0.1 to 0.2 ml). Rats were killed at the indicated times and exudates collected by pleural lavage. Exudates were then processed and assayed for protein, cell and histamine contents as described in the previous report (Z01 HL 00956-02 LCP). In experiments involving mice, drug concentrations were adjusted to give the same dose/kg as in the rats. The injection volumes were reduced to 0.05 ml for the intrapleural injection and 0.2 ml for the subcutaneous injections. In addition, the pleural cavity was washed with 0.2 ml of in Dulbecco's phosphate buffered saline which contained 5 mM EDTA (without CA++ and Mg++).

Major Findings: Differences in the inflammatory response to dextran and dextran-SO_4. Dextran T10 induced only fluid accumulation (1.5 + 0.30 ml vs 0.05 + 0.02 in saline injected rats), whereas dextran-SO_4 induced both fluid and protein infiltration (fluid volume: 0.68 + 0.12 ml; protein content: 38 + 9 mg). The accumulation of fluid in response to neither dextran nor dextran-SO_4 was affected by mepyramine (5 mg/kg) and metiamide (100 mg/kg) given in combination or by injection of indomethacin (5 mg/kg i.v.). However, in contrast to fluid accumulation extravasation of plasma protein induced by dextran-SO_4 was totally (100%) suppressed by the combination of antihistamines or indomethacin. The results suggested that fluid accumulation in response to the dextran T10 and dextran-SO_4 was not a consequence of histamine release but may be due to a nonspecific effect (for example, osmosis). Protein accumulation in response to dextran-SO_4 did appear to be a consequence of histamine release.

Dynamics of water exchange in the pleural cavity. The injection of ^3H_2O along with saline or dextran T10 solution (0.2 ml or 1 ml) into the pleural cavity was followed by rapid loss (t-1/2 <3 min) of ^3H from the cavity. Rate of loss was the same irrespective of volume of solution injected. The data illustrate the rapidity of exchange of water molecules between pleural cavity and circulation. Injection of hyperosmotic solution should therefore lead to rapid adjustment of water volumes in the pleural space.

Studies with mast cell-deficient mice ($WBB6F_1/J$-w/w^v). Few mast cells were found in the pleural and peritoneal washes of w/w^v mice. Mast cells constituted 1 + 0.3% and 6 + 1% of cells harvested respectively from the pleural and peritoneal cavity of the congeneic normal mice (+/+ mice). Macrophages and lymphocytes constituted the remaining cells. There were essentially no eosinophiles or neutrophils. Although the total amount of cellular histamine found in the peritoneal washes of both groups of mice differ significantly (129 + 9 ng, n=8 and 7 + 1 ng; n=8 for +/+ and w/w^v mice, respectively), the volume of fluid accumulated in the pleural cavity of either group was similar upon the injection of 7.5 mg dextran T10: 0.29 + 0.02 ml for the +/+ mice and 0.32 + 0.03 ml for the w/w^v mice. Furthermore, the exudate was devoid of plasma protein and cells. We have previously found that, in rat, the intrapleural injection of anti-IgE or compound 48/80 resulted in rapid accumulation of fluid, plasma proteins and neutrophils and that the responses were generally correlated with the extent of mast cell degranulation and histamine release. Therefore, the fluid accumulation in response to dextran T10 was probably due to osmosis and the partial mast cell degranulation maybe a consequence of osmotic shock.

Characteristics of the inflammatory response to dextran sulfate. The above studies with dextran sulfate suggested that whereas fluid accumulation may be a consequence of osmosis, protein and cell infiltration might be due to release of histamine and/or arachidonic acid metabolites. Following injection of dextran sulfate, almost all mast cells in the exudate were degranulated as indicated by microscopic examination. Dextran sulfate is a known activater of complement 3 (R. Burger et al. Immunology 29:549, 1975), and C3a is a well-established stimulant of mast cell degranulation. The ability to block completely the response to dextran-SO_4 with antihistamines and indomethacin noted above could indicate that C3a, if involved, acts through release of secondary mediators.

Significance to Biomedical Research and the Program of the Institute: In past and present studies experimentally induced inflammations (in rats and mice) of varying degree of complexity have been studied in terms of the composition of inflammatory exudates and specific mediator(s) involved. The study indicates: 1) No known mediator is associated with the dextran T10 induced transudation; 2) histamine is the predominant mediator of responses to compound 48/80 and anti-IgE; 3) C3a, histamine and perhaps arachidonate metabolites of mast cell origin are probable mediators in the dextran-SO_4 model; and 4) arachidonate metabolites of nonmast cell origin are the primary mediators in the carrageenan model. The findings point to the multiple pathways available for initiating inflammatory responses.

Proposed Course of Project: The concentration of C3a and C5a in the exudate collected from dextran-SO_4 treated rats will be determined. The recently available radioimmunoassay kits (Upjohn Diagnostics) for human C3a and C5a should provide a highly sensitive assay for these complement components. Experiments will be performed to check whether or not the kits can be used in the assay for complement components in rats. Should the antibodies against human C3a and C5a not cross react with rat C3a and C5a, inhibitors of the complement activation cascade will be used to assess the role of C3a in the dextran-SO_4 induced inflammation. As the current studies have defined the multiple pathways for inflammatory response *in vivo* with classic inflammatory agents, future studies will

focus on the effect of these agents on specific cell types *in vitro* as outlined in project report no. Z01 HL 00617-08. Current project is terminated.

Publications: None

3-P

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00958-02 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Drug-induced cardiotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Vilas Shirhatti	Vist. Fellow	LCP	NHLBI
Others:				
	M. George	Guest Worker	LCP	NHLBI
	G. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcium ionophore, A23187 induced a dose dependent (IC50-5μM) cardiotoxicity in cardiac myocytes in culture. A 23187 also markedly potentiated the toxicity induced by daunomycin. This effect was seen both as a leakage of enzyme as well as in the reduction of ATP as well as leakage of[14]C-AMP in cells which have been labeled with [14]C-adenine. Electron-microscopic studies of these cells indicate the primary damage occurred in the mitochondria with little effect on the myofibrils. This early effect of ionophore and daunomycin was also seen on the beating rate which was markedly reduced in the cardiac myocytes. Verapamil, calcium channel blocker, potentiates the toxicity while another calcium channel blocker, nifedepine had no effect. Amrinone and milrinone which are bipyridine ionotropes markedly decreased the toxicity caused by daunomycin as determined by a decrease in the loss of ATP and enzyme leakage. Milrinone was found to be superior in this respect. Cyclic AMP has been implicated in the ionotropic effect as well as the cardiotoxicity induced by catecholamines. HL 725, a potent phosphodiesterase inhibitor, was found to have no protective effect on daunomycin-induced cardiotoxicity.

Project Description:

Objectives: A number of anthracyclins such as adriamycin and daunamycin have been developed as effective antitumor agents against leukemia as well as various solid tumors. However, they induce cardiac myopathies in humans in a dose and time dependent fashion. Even though a number of mechanisms have been proposed, none have been experimentally proven. One of the main difficulties has been that in order to induce the disease in animals in a predictable manner the drugs have to be administered to animals over a long period of time. Both the Zbinden rat model and the rabbit model have been accepted as in vivo model for the induction of cardic myopathies by anthracyclin drugs, but neither have been proven as an ideal model for study of the mechanism. Last year we reported the development of a cardiac myocyte culture which could be employed as an effective model for such studies. We have utilized this model for the study of the role of calcium in adriamycin and daunomycin induced cardiotoxicity and to investigate the ability of a number of calcium channel blockers as well as other drugs that would greatly reduce or abolish cardiotoxicity.

Methods Employed: The method of isolation and culture of neonatal rat cardiac myocytes were described in the last project report. We have modified the cell isolation technique, which greatly enhances the yield and quality of cells, and the time required for isolation has been greatly reduced (as much as 90%). The cells have been cultured in a newly developed plastic flask which markedly increases the plating efficiency and growth and differentiation of myocytes.

The leakage of various enzyme such as lactic acid dehydrogenase (LDH), creatine phosphate kinase (CPK), reduction of ATP and leakage of ^{14}C-AMP have been employed for monitoring cell damage along with phase contrast as well as electron microscopy.

Major Findings: Calcium ionophore, A23187 induced a dose dependent (IC_{50}-5μM) cardiotoxicity in cardiac myocytes in culture. A 23187 also markedly potentiated the toxicity induced by daunomycin. This effect was seen both as a leakage of enzyme as well as in the reduction of ATP as well as leakage of ^{14}C-AMP in cells which have been labeled with ^{14}C-adenine. Electron-microscopic studies of these cells indicate the primary damage occurred in the mitochondria with little effect on the myofibrils. This early effect of ionophore and daunomycin was also seen on the beating rate which was markedly reduced in the cardiac myocytes. Verapamil, calcium channel blocker, potentiates the toxicity while another calcium channel blocker, nifedepine had no effect. Amrinone and milrinone which are bipyridine ionotropes markedly decreased the toxicity caused by daunomycin as determined by a decrease in the loss of ATP and enzyme leakage. Milrinone was found to be superior in this respect. Cyclic AMP has been implicated in the ionotropic effect as well as the cardiotoxicity induced by catecholamines. HL 725, a potent phosphodiesterase inhibitor, was found to have no protective effect on daunomycin-induced cardiotoxicity.

Significance to Biomedical Research and Program of the Institute: The development of a cell culture model for the study of a mechanism of cardiotoxicity should greatly enhance our attempt to understand the mechanism of toxicity of drugs to the heart and thus enable us to develop drugs which are potentially less toxic to the heart without impairing their ability to kill cancer cells.

Proposed Course of Project: We propose to examine a number of drugs of the type of milrinone which are more potent inhibitors of the toxicity of adriamycin and daunomycin. During the studies of various analogs of adriamycin, we have found a single analog, anthracinedione which appears to have less toxicity in the cardiac myocytes in cultures. A number of analogs of this will be examined in order to select a few which will be effective for studies in vivo. We are also planning to covalently link adriamcyin and daunomycin and their analogues to various micro beads in order to determine whether they are capable of killing cancer cells without having an effect on the heart cells.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00962-02 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological studies on the mechanism of halothane-induced hepatotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation)

P.I.: Hiroko Satoh — Vist. Assoc. — LCP — NHLBI

Others:

Lance R. Pohl — Section Chief — LCP — NHLBI

James R. Gillette — Chief — LCP — NHLBI

COOPERATING UNITS (if any)

Dr. Yu Fukuda and Dr. V.J. Ferrans, Ultrastructure Section, Pathology Branch, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The fulminant hepatoxicity caused by halothane has been thought to have an immunological basis because this toxicity occurs most often after repeated administration of halothane and because sera from patients recovering from severe halothane hepatoxicity have been found to contain antibodies that bind to the surface membranes of hepatocytes of rabbits treated with halothane. In order to determine whether halothane's major reactive metabolite, trifluoroacetyl halide (CF_3COX) results in covalent binding of trifluoroacetyl (TFA) groups to hepatocytes, we have developed specific and sensitive peroxidase enzyme-linked immunosorbent assays (ELISA) and an indirect immunofluorescence staining method for identifying TFA-hepatocytes. Liver sections prepared from rats at 4 hr after halothane administration were stained preferentially in the centrilobular region with anti TFA antiserum. The specificity of the assay for the TFA group was confirmed by the complete inhibition of the staining by TFA-Llysine. Moreover, treatment of rat with deuterated halothane resulted in significantly less staining than did halothane. Twenty four hours after halothane administration hepatocytes were isolated and stained by indirect immunofluorescence. The immunofluorescence showed a linear or a granular pattern on the surface membranes. These results indicate that CF_3COX either reacts directly with plasma membrane or with other cellular components which become incorporated into the plasma membrane. In order to study further the immunological mechanism of halothane hepatitis, we intend to determine what component is modified by TFA, and how these altered hepatocyte membranes can initiate an immune response, which can result in hepatocellular damage. We also plan to develop an animal model of halothane hepatitis to evaluate the immunological mechanism, and to study this disease in greater detail. The immunological methodologies that will be developed in these studies may serve as a model for investigating the mechanism of other drug induced toxicities which are believed to have an immunological origin. In susceptible individuals these membrane changes may lead to sensitization and damaging immune reactions.

3312

Project Description:

Objectives: The intent of this investigation is to determine the molecular basis of halothane-induced hepatotoxicity. Experiments with specific antiserum will be made to determine whether this toxicity is caused by sensitization to trifluoroacetylated hepatocytes.

Method Employed: 1. Specific antibodies against TFA lysyl groups were prepared by immunization of rabbits with TFA rabbit serum albumin (TFA-RSA). 2. Radioimmunoassay was used to determine the titers of antisera. 3. Microtiter peroxidase ELISA (enzyme linked immunosorbent assay) was used to characterize anti TFA-RSA serum. 4. Utilizing anti-TFA-RSA serum, peroxidase ELISA tissue staining, Western blot staining of solubilized tissue and indirect immunofluorescent antibody staining of hepatocytes have been done to detect TFA bound adduct in the halothane treated hepatocytes from rats. 5. Deuterated halothane was used in place of halothane to confirm the specificity of the anti-TFA-RSA staining.

Major Findings: 1. Anti-TFA-RSA serum is specific to TFA-lysyl group and does not react against reductive metabolites of halothane. 2.TFA hepatocytes localized preferentially in centrilobular region of liver. 3. Western blott staining of halothane liver tissue revealed five TFA-fractions. The major component (53,000 Daltons) appeared to be identical to a TFA-fraction observed in the microsomes and possibly is a TFA cytochrome P-450. 4. Immunofluorescence analysis of intact hepatocytes showed that TFA components were also present on the outer surface of hepatocyte membrane. 5. Deuterated halothane administration in place of halothane results in significantly less binding of TFA group in the liver.

Significance to Biomedical Research and Program of the Institute: Halothane-induced hepatotoxicity which is often fatal in humans has been suggested to have an immunological basis. However, the mechanism is poorly known. Halothane is metabolized oxidatively in the liver by cytochrome P-450 to trifluoroacetyl chloride, which binds covalently to liver macromolecules. We have now obtained immunochemical evidence of trifluoroacetylated components within the cell and on cell surfaces. TFA altered hepatocytes may lead to sensitization and damaging immune reaction in susceptible individuals. The methods that we have used and will develop in the future should serve as a model for investigating the mechanism of other drug-induced toxicities which are believed to have an immunological origin.

Proposed Course of Project: In order to determine in greater detail the mechanism of halothane-induced hepatotoxicity, we intend to identify the major cellular component that is modified by trifluoroacetylation. We also intend to obtain hepatocytes from patients exposed to halothane and to determine whether these cells bind the antibody and to determine whether patients with halothane hepatotoxicity have antibodies or specific lymphocytes in their serum directed against TFA groups or TFA altered membrane components on their own hepatocytes.

If we can find such antibodies or lymphocytes, we will try to determine the mechanism of the immunotoxicity. Furthermore, we intend to develop an animal model of this immunotoxicity in mice which are susceptible to autoimmunity so that we will be able to clarify how drug altered autocomponents can be recognized as antigen by the immune system.

Publications: None

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

OFFICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 00967-02 LCP

October 1, 1983 through September 30, 1984

Regulation of cytochrome P-450 turnover

P.I. Helen Davies	Staff Fellow	LCP	NHLBI
Others:			
Lance R. Pohl	Section Chief	LCP	NHLBI
Hiroko Satoh	Vist. Assoc.	LCP	NHLBI
John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Pharmacological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cytochrome P-450 is a family of isozymes that are present in most tissues of the body and are involved in the metabolism and activity of steroids, prostaglandins, leucotrienes, fatty acids, drugs and environmental chemicals. It has recently been shown that several of these isozymes turnover at different rates, but the basis for these findings is not understood. In this study we have found that during the catalytic turnover of rat liver microsomal cytochrome P-450 in vitro that a selective degradation of the isozymes occurred. Most striking were the findings of covalently bound heme fragments to apoprotein and high molecular weight derivatives of the apoproteins. We believe that this may represent the selective "tagging" of the apocytochrome P-450 by heme fragments or other cellular components that signals the catabolic enzymes to hydrolyze this group of enzymes.

3 5 5

Project Description:

Objectives: To determine the pathway for the in vivo turnover of cytochromes P-450 heme and apoprotein and to relate the findings to further our understanding of the regulation of cellular turnover.

Methods Employed: Incubations of rat liver microsomes, containing radiolabeled heme or protein, have been carried out under conditions in which P-450 loss is observed. Resultant covalent binding to protein of heme fragments was determined by precipitation of the protein in acidic solvents, under conditions where noncovalently bound heme is released. Distribution of the bound labeled heme fragments among microsomal proteins was determined by subjecting the microsomes, following the incubation, to SDS polyacrylamide gel electrophoresis, sectioning the gels, and measuring the radioactivity in the gel sections. Similarly, molecular weight changes in microsomal proteins accompanying P-450 degradation were determined by electrophoresis of microsomes which contained radiolabeled proteins. Changes in the levels of individual P-450 isozymes in incubated microsomes were determined by separating the isozymes by anion exchange chromatography. Distribution of the different forms of P-450 among the chromatography peaks was determined by electrophoresis.

Major Findings: Substantial loss of cytochrome P-450 was observed in rat liver microsomes incubated in the presence of NADPH, CCl_4 and NADPH, or linoleic acid hydroperoxide. The P-450 loss was accompanied by irreversible covalent binding of heme fragments to microsomal proteins. Electrophoretic separation of the microsomal proteins showed that the covalent binding was specific for proteins in the P-450 molecular weight region and in the very high molecular weight region, where polymers of P-450 would be found. Electrophoresis of microsomal incubation mixtures containing radiolabeled protein, showed that the loss of protein in the P-450 molecular weight region accompanying spectrally measured P-450 loss was nearly balanced by a gain of protein in the very high molecular weight region. When the P-450s in the incubated microsomes were separated by anion exchange chromatography it was seen that there were losses in all the forms of P-450. Selectivity was also seen, however, in that destruction of some forms of P-450 was proportional to total P-450 loss while other forms were degraded to the same extent in all cases, suggesting differing stabilities of these forms. Covalent binding of heme fragments to P-450 apoprotein was also detected in the chromatography fractions. In two cases binding was proportional to P-450 loss in that peak. In a third peak binding did not reflect P-450 loss, which was probably accounted by a heme-labeled protein which eluted late from the column, possibly as P-450 polymers. The most striking finding was the similar qualitative nature of the P-450 destruction in the three types of incubations, suggesting a common mechanism of P-450 loss.

Significance to Biomedical Research and Program of the Institute: Cytochrome P-450 is found in most tissues of the body. This enzyme exists in multiple forms with different specificities and is involved in the metabolism of steroids prostaglandins, leucotrienes, fatty acids, drugs and environmental chemicals. It has recently been shown that several isozymes turnover at different rates. It is important to understand the molecular basis for these differences because factors affecting turnover can affect the steady-state concentration and overall activity

of this important group of enzymes. Reports in the literature suggest that the in vivo turnover of the heme of cytochrome P-450 proceeds by a pathway outside of the known heme metabolizing pathway (the heme oxygenase system). These pathways, however, have not been fully characterized. We have found that degradation of cytochrome P-450 in vitro under three different types of incubation conditions, leads to the covalent binding of P-450 heme fragments and possibly other cellular components to P-450 apoprotein. If this covalent binding occurs in vivo it may "tag" the apoprotein for degradation by catabolizing enzymes. Since we have observed differences among the P-450 forms both in the susceptibility to degradation in in vitro incubations and in some properties of the resultant heme-labeled apoprotein, it may be that these differences in the heme breakdown (and/or susceptibility to breakdown are the cause of P-450 turnover rate differences. Understanding the mechanism of the heme covalent binding in various P-450 forms may therefore provide insight into factors affecting turnover. Unknown soluble heme fragments are also released in in vitro heme degradations. These may also turn out to be important regulators of P-450 levels.

Proposed Course of Project: We intend to do the following: 1) Identify the structure of the soluble and bound heme fragments that are produced during P-450 loss in vitro. This should probably help us explain the mechanism of the P-450 loss in vitro. If the same products are observed in vivo this would strongly indicate that the same mechanism of P-450 loss occurs in vivo. 2) We want to determine whether the "tagging" of the P-450 by heme fragments leads to the turnover by the catabolizing enzymes in the body. This problem will be approached by developing immunochemical assays for the P-450s and their "tagged" derivatives. We then will determine both in vitro and in vivo whether the "tagged" proteins turnover faster than the untagged enzymes. 3) To the best of our knowledge, no one has yet identified the catabolizing proteins that degrade P-450. We intend to identify and characterize these enzymes because they likely have an important role in the regulation of the steady levels of cytochrome P-450.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00968-02 LCP

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanism of extrahepatic bromobenzene toxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
Other Investigator: Serrine S. Lau	Vist. Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously shown that o-bromophenol and 2-bromohydroquinone, metabolites of bromobenzene, are nephrotoxic. Both o-bromophenol and 2-bromohydroquinone are converted to several isomeric GSH conjugates which give rise to covalently bound material in vitro. Moreover, these GSH conjugates cause elevations in BUN levels and cause nephrotoxicity similar to that observed with bromobenzene, o-bromophenol and 2-bromohydroquinone. Inhibition of γ-glutamyl transpeptidase inhibited the covalent binding of the GSH conjugates and the nephrotoxicity of 2-bromohydroquinone. 2-BHQ cysteine but not N-acetyl cysteine conjugates were nephrotoxic.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives: Treatment of rats or mice with a single i.p. dose of bromobenzene (9.3 mmol/kg) has been shown to produce necrosis of the proximal convoluted renal tubules. The production of this renal necrosis was associated with the covalent binding of radiolabeled material to kidney protein. Studies in mice on the metabolism and covalent binding of ^{14}C-bromobenzene in vitro suggested that the renal necrosis was caused by a metabolite formed in the liver and transported by blood to the renal tubules. The nature of this proposed metabolite was unclear.

We recently demonstrated that o-bromophenol and 2-bromohydroquinone, metabolites of bromobenzene, were nephrotoxic in rats. Since this mechanism of toxicity of these metabolites remained obscure the present experiments were designed to elucidate these problems.

Methods Employed: o-Bromophenol and 2-bromohydroquinone were incubated with rat liver microsomes in the presence of 1 mM ^{35}S-glutathione. The resulting isomeric ^{35}S-GSH conjugates were isolated and purified by HPLC and incubated with 10,000 x g supernatant of rat liver and kidney in the presence and absence of AT-125 (Acivicin; NSL 16350) an inhibitor of γ-glutamyl transpeptidase. GSH cysteine and N-acetylcysteine conjugates of 2-bromohydroquinone were chemically synthesized and were given to male Sprague-Dawley rats (150-170 g) by i.v. injection. Renal damage was assesed by determination of BUN levels and by microscopic examination of kidney sections stained with eosin and hematoxylin.

Major Findings: Incubation of either o-bromophenol or 2-bromohydroquinone with rat liver microsomes and 1 mM ^{35}S-GSH gave rise to several ^{35}S-GSH adducts. Incubation of these conjugates with 10,000 x g supernatants of rat liver or kidney gave rise to covalently bound material. Covalent binding to kidney was 1-2 times greater than that to liver homogenate. Injection of the 2-bromohydroquinone-GSH conjugates intravenously to rats caused substantial elevations in BUN and histopathological changes in kidney sections that were identical to those found in bromobenzene, o-bromophenol or 2-bromohydroquinone treated rats. AT-125 inhibited the covalent binding of ^{35}S-2-bromohydroquinone conjugates to kidney supernatant by 35% and decreased 2-bromohydroquinone (0.93 mmol/kg i.p.) mediated elevations in BUN. Injection of 2-bromohydroquinone cysteine conjugates to rats caused elevations in BUN and histopathological changes in kidney sections that were identical to those obtained in rats treated with glutathione-bromohydroquinone. In contrast, injection of N-acetyl cysteinyl bromohydroquinone neither elevated BUN nor caused any histopathological alterations in the kidney.

Significance to Biomedical Research and Program of the Institute: Conjugates of cysteine not of N-acetyl cysteine are substrates for CYS-conjugate β-lyase. Moreover it appears that all of the substrates for β-lyase generate reactive thiol species that eventually inactivate the enzyme. The data therefore suggest that hydrolysis of BHQ-GSH to BHQ-CYS followed by activation by kidney β-lyase, may be the pathway of nephrotoxicity.

Proposed Course of Project: Terminated

Publications:

Lau, S.S., Monks, T.J., Greene, K.E. and Gillette, J.R.: The role of o-bromophenol in the nephrotoxicity of bromobenzene. Toxicol. Appl. Pharm. 72: 539-549, 1984.

Lau, S.S. Monks, T.J. and Gillette, J.R.: Identification of 2-bromohydroquinone as a metaboltie of bromobenzene: Implications for bromobenzene induced nephrotoxicity. J. Pharmacol. Exp. Ther., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00960-01 LCP

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Formation of propranolol glutathione conjugates by microsomes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Henry A. Sasame	Chemist	LCP	NHLBI
Other: James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Formation of glutathione conjugates of propranolol in situ by guinea pig microsomes supplemented with GSH-S-transferase and GSH was based on two criteria. 1) After treatment with α-glutamyltranspeptidase, the glutathione conjugate detected in a HPLC system was converted to another substance having a shorter retention time than that treated with BSA. 2) Double label experiment using ^{14}C-GSH and ^{3}H propranolol [4-^{3}H] demonstrated unequivocally that the conjugate peak detected in the HPLC system is glutathione conjugate. Since we have previously found that the decrease in the formation of 5OHPr correlated with the formation of a GSH-conjugate in liver microsomes from BNF treated rats, it seems likely that the GSH conjugate and 5OHPr are derived from a common intermediate, perhaps propranolol-5,6-oxide. It is noteworthy that the presence of GSH plus GSH-transferase caused the lowering of 5-hydroxypropranolol formation in all microsomes from stomach, small intestine and colon, suggesting an important role of GSH-transferase in GI tract for the inactivation of the epoxide.

Project Description:

Objectives: Two years ago we reported that in the presence of soluble fraction plus GSH, there was a concomitant decrease in the 5OHPr formation and an increase in the formation of a water soluble metabolite of propranolol by liver microsomes from BNF-treated rats. Subsequently, during the course of cross-reactivity studies with a monoclonal antibody raised against a cytochrome P-450 induced by 3-methylcholanthrene, we have discovered that the rate of formation of 5OHPr in guinea pig microsomes was the highest among all animal species we have examined. Thus in order to study the nature of this conjugate we decided to use guinea pig microsomes.

Methods Employed: ^{14}C-GSH was prepared as follows: Oxidized ^{14}C-GSSG was reduced in situ with a glutathione reductase plus unlabeled GSSG and a NADPH generating system. The extent of reduction was assayed colormetrically using unlabeled reduced GSH as a standard. The propranolol glutathione conjugate and other propranolol metabolites were assayed by HPLC. GSH transferase was partially purified by the method of Jacoby from the soluble fraction of liver of phenobarbital treated rats.

Major Findings: 1) The formation of the glutathione conjugate of propranolol requires the presence of the GSH-transferase and GSH.

2) The treatment of the GSH-conjugate with γ-glutamyl transpeptidase resulted in a shift of retention time from 9.5 min to 7.5 min in HPLC system in which the mobile phase consisted of 12.5% MEOH, 1% HPLC and 87.5% H_2O. But the treatment of the conjugate with BSA caused no such shift in its retention time.

3) When ^{3}H-propranolol and ^{14}C-GSH were incubated with microsomes from the stomach, small intestine and colon there was a decrease in the 5OHPr peak and an increase in a peak containing both labels. This strongly suggests that an intermediate metabolite formed in situ could be converted to the GSH conjugate.

4) The level of GSH transferase activities in liver, stomach, small intestine, and colon BNF treated rats were 24, 3, 3 and 1, respectively. The low level of GSH-transferase in colon may contribute to the prevalence of carcinogenesis in the colon.

Significnce to Biomedical Research to Program of the Institute. Though propranolol is known to be a safe drug it is worth while to pursue the effect of various inducers on both the rate of formation of its GSH-conjugate and level of GSH-transferase in GI tract using propranolol as a model compound.

Proposed Course of Project: In view of the current concensus that carcinogenesis in colon is likely to be due to prolonged exposure to toxic substances, it is of interest to investigate the roles of GSH-transferase in GI tracts of animals exposed to various chemicals.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00970-01 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

PROJECT TITLE (Must fit on one line between the borders.)

Mechanism of induction of cytochrome P-450: An in vitro model

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Vilas Shirhatti	Vist. Fellow	LCP	NHLBI
Others:			
C.T. Liu	Chemist	LCP	NHLBI
G. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A number of experiments were performed in order to arrive at a combination of additives for the maintenance of cytochrome P-450 in liver cells in culture at original level. A combination of 50 μM δ-aminolevulinic acid (ALA), 5 μM hydrocortisone, 1 μM dexamethasone, and 10% Nu Serum in Williams E medium proved to be the most satisfactory in maintaining cytochrome P-450 isozymes at 80-90% of original levels. Even though dexamethasone and hydrocortisone induced other proteins as shown by electrophoresis, the P-450 isozyme pattern of liver appears to be maintained as in the original cells. This is true with control as well as phenobarbital induced cytochrome P-450. [35]S-methionine was incorporated into a number of proteins in the cells. At present we are purifying the different forms by DEAE chromatography and SDS gel electrophoresis after precipitation with specific antibodies to cytochrome P-450. We have raised several monoclonal antibodies against these isozymes and these will be utilized for purification of [35]S-labeled cytochrome P-450's in cells both in the control as well as induced state. The monoclonal antibodies against cytochrome P-450 are being characterized.

Project Description:

Objectives: One of the main objectives of these studies has been to establish an in vitro liver cell culture system and methods for studying the induction of cytochrome p-450 isozymes by phenobarbital. Even though it has been possible to induce cytochrome P-450 isozyme in vivo in animals it has not been possible to do so in cells in culture. One of the difficulties has been the inability to maintain cytochrome P-450 levels in cells at original levels for any extended period of time. The reason for the loss of cytochrome P-450 is unknown. Various attempts have been made to maintain cytochrome P-450 but with little success. Since one of long term objective has been to study the mechanism by which phenobarbital induces cytochrome P-450 isozymes, we have undertaken a detailed study of required media components in order to maintain the enzyme at the original level. Moreover, we have examined methods which would be useful for monitoring the synthesis of these enzymes in vitro so it could be applied towards the study of phenobarbital induction.

Methods Employed: Methods for isolation of liver cells and culturing them have been reported previous project reports. The main modification has been the replacement of horse serum by Nu serum (Collaborative Research Inc.) which consists of a mixture of growth factors with very little serum protein. This has markedly improved the culture conditions. The cells were cultured in Williams E medium containing to 10% Nu Serum on collagen coated petri dishes. Cytochrome P-450 was measured after solubilization of the cells with emulgen and sodium cholate containing buffer in the presence of 20% glycerol. The pattern of cytochrome P-450 isozymes in the cells were examined by SDS-gel electrophoresis. The rate of enzyme (P-450) synthesis was monitored by prelabeling the protein with ^{35}S-methionine in a medium containing low methionine. Cytochrome P-450 containing ^{35}S-methionine was isolated by specific precipitation with antibodies and isolation of the isozyme by DEAE chromatography and SDS electrophoresis. We have prepared several of monoclonal antibodies and these will be used for the characterization of isozymes of cytochrome P-450.

Major Findings: A number of experiments were performed in order to arrive at a combination of additives for the maintenance of cytochrome P-450 in liver cells in culture at original level. A combination of 50 µM δ-aminolevulinic acid (ALA), 5 µM hydrocortisone, 1 µM dexamethasone, and 10% Nu Serum in Williams E medium proved to be the most satisfactory in maintaining cytochrome P-450 isozymes at 80-90% of original levels. Even though dexamethasone and hydrocortisone induced other proteins as shown by electrophoresis, the P-450 isozyme pattern of liver appears to be maintained as in the original cells. This is true with control as well as phenobarbital induced cytochrome P-450. ^{35}S-methionine was incorporated into a number of proteins in the cells. At present we are purifying the different forms by DEAE chromatography and SDS gel electrophoresis after precipitation with specific antibodies to cytochrome P-450. We have raised several monoclonal antibodies against these isozymes and these will be utilized for purification of ^{35}S-labeled cytochrome P-450's in cells both in the control as well as induced state. The monoclonal antibodies against cytochrome P-450 are being characterized.

Significance to Biomedical Research and the Program of the Institute. Since one of the major stumbling block namely maintenance of cytochrome P-450 has been overcome, the liver cell culture model should greatly enhance the study of the mechanism of induction of phenobarbital. Since phenobarbital has not been able to induce cytochrome P-450 in vitro in liver cells, the study of the required factors which are essential for its induction would greatly enhance the chances for the understanding of the mechanism of the induction of these class of proteins.

Proposed Course of Project: Since our studies have indicated that Nu Serum which contains very little serum proteins but contains selenium is able to maintain cytochrome P-450 and since recent findings by others have indicated the requirement for the induction of cytochrome P-450 by phenobarbital, we are undertaking the study of the role of selenium in the induction of enzyme by phenobarbital. It appears that phenobarbital is able to induce the apoenzyme but not able to form holoenzyme. The requirement for the introduction of heme to the apoenzyme will also be examined. Asorbic acid has been known to play a role and this will be studied in detail in the liver cell model. We have isolated a number of monoclonal antibodies against cytochrome P-450 isozyme and these will be used for the isolation and characterization for the form induced by phenobarbital in vitro.

Publication:

Vulliemoz, Y., Triner, L., Verosky, M., Hamm, M.W. and Krishna, G.: Deuterated halothane - Anesthetic potency, anticonvulsant activity, and effect on cerebellar cyclic guanosine 3',5'-monophosphate. Anesthesiology and Analg. 63: 495-498, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF ANNUAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00971-01 LCP

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Decreases in cytochrome P-450 caused by tunicamycin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation.)

PI:	Yogendra Singh	Visiting Fellow	LCP	NHLBI
Other Investigators:				
	C.T. Liu	Chemist	LCP	NHLBI
	V. Shirhatti	Vist.Fellow	LCP	NHLBI
	G. Krishna	Section Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tunicamycin caused marked decreases in liver cytochrome P-450 levels in a dose-dependent fashion but only a small effect (15% at 50 µg/kg) decreased in cytochrome c reductase activity 50 µg/kg decreased cytochrome P-450 by 50%. Whereas 100 µm/kg tunicamycin decreases by 80%. Since higher doses caused more than 50% mortality, 50 µg/kg dose was used for all experiments. The reduction of cytochrome P-450 was much more marked with control animals than phenobarbital-induced animals. Tunicamycin did not lower cytochrome P-450 within 24 hrs and required three daily doses to cause a decrease in cytochrome P-450.

Since tunicamycin caused a marked decrease in food and water intake, the effect of tunicamycin was examined in animals which were either deprived of food and water or food alone. The effect of food and water deprivation on cytochrome P-450 was minimal and tunicamycin still caused a marked reduction in the cytochrome P-450 in these animals indicating the loss of cytochrome P-450 by tunicamycin was not due to suppression of food and water intake. The effect of tunicamycin on the glycosylation of cytochrome P-450 is being examined. Electrophoresis of microsomal protein of tunicamycin treated rats indicated a general loss of cytochrome P-450 apoprotein in the microsomes with a concomittant loss of the cytochrome P-450 levels. Antibody staining of the gels after Western blotting also indicated a great loss of apoproteins. The mechanism by which tunicamycin induced a specific loss of cytochrome P-450 remains obscure.

Project Description:

Objectives: The term cytochrome P-450 refers to a mixture of structurally related hemoproteins which are involved in the metabolism of various other drugs and xenobiotics. The isozymes of cytochrome P-450 are products of separate genes which are induced or suppressed to varying degree by a number of compounds. Even though the mechanism by which various forms are induced is known to some degree, the mechanism by which these isozymes are suppressed are not clearly understood. It is possible that post translation modifications such as acylation, phosphorylation and glycosylation may affect the stability of cytochrome P-450. The role of such modification in the degradation of proteins is not completely understood. A number of cytochromes P-450 isozymes has been shown to be partially glycosylated, but the role of glycosylation in the stability of the enzyme is not known. Even though the glycosylation on cytochrome P-450 occurs only partially, such partial glycosylation appears to occur in rhodopsun. For this reason we have investigated the role of glycosylation in the maintenance of cytochrome P-450 isozyme in the liver in vivo by specific inhibition of glycosylation by tunicamycin. Tunicamycin, which is an antibiotic isolated from culture of Streptomycis lysosuperticous has been shown to inhibit glycosylation by inhibiting the first step involving dolichol phosphate and UDP N-acetyl glycosamine condensation by N-acetyl glycosamine dolicol phosphate transferase.

Methods Employed: Male Sprague Dawley rats weighing 160-200 g obtained from Taconic Farms New York, and were maintained with food and water ad libitum. The rats were injected i.p. daily for 3 days with varying concentrations of tunicamycin dissolved in 3.3% DMSO in water. Control rats received the same solvent without the drug. Phenobarbital was administered at a dose of 80 mg/kg daily for 3 days, with or without tunicamycin. In some experiments rats were fasted and injected with tunicamycin. In some cases food and water were removed from rats during the tunicamycin treatment. Appropriate controls were included in all cases. Cytochrome P-450 was measured in the microsomes after solubilization with emulgen buffer by the method of Omura and Sato (J.Biol.Chem. 239:2379-85,1964). Cytochrome c reductase was determined by the rate of cytochrome c disappearance in the presence of NADPH. The isozyme pattern was examined by SDS-PAGE. The gels were either stained by commossie blue or gels were transferred to nitrocellulose paper and stained immunochemically with a specific antibody against cytochrome P-450.

Major Findings: Tunicamycin caused marked decreases in liver cytochrome P-450 levels in a dose-dependent fashion but only a small effect (15% at 50 µg/kg) decreased in cytochrome c reductase activity 50 µg/kg decreased cytochrome P-450 by 50%. Whereas 100 µm/kg tunicamycin decreases by 80%. Since higher doses caused more than 50% mortality, 50 µg/kg dose was used for all experiments. The reduction of cytochrome P-450 was much more marked with control animals than phenobarbital-induced animals. Tunicamycin did not lower cytochrome P-450 within 24 hrs and required three daily doses to cause a decrease in cytochrome P-450.

Since tunicamycin caused a marked decrease in food and water intake, the effect of tunicamycin was examined in animals which were either deprived of food and water or food alone. The effect of food and water deprivation on cytochrome P-450 was minimal and tunicamycin still caused a marked reduction in the cytochrome P-450 in these animals indicating the loss of cytochrome P-450 by tunicamycin was not due to

suppression of food and water intake. The effect of tunicamycin on the glycosylation of cytochrome P-450 is being examined. Electrophoresis of microsomal protein of tunicamycin treated rats indicated a general loss of cytochrome P-450 apoprotein in the microsomes with a concomittant loss of the cytochrome P-450 levels. Antibody staining of the gels after Western blotting also indicated a great loss of apoproteins. The mechanism by which tunicamycin induced a specific loss of cytochrome P-450 remains obscure.

Significance to Biomedical Research to Program of the Institute: Tunicamycin has been thought to be a specific inhibitor of glycosylation of proteins. If glycosylation of cytochrome P-450 occurs, this drug could be used for probing the role it might play in the degradation of cytochrome P-450. The dose of tunicamycin to inhibit glycosylation of other proteins will be compared with that required to inhibit cytochrome P-450.

Proposed Course of Project: The mechanism by which tunicamycin inhibits the synthesis or enhances degradation of cytochrome P-450 will be investigated by ^{35}S-methionine-labelling and isolation of cytochrome P450 by specific antibody precipitation followed by SDS-PAGE.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00972-01 LCP

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of inactivation of biogenic amines by microvascular endothelial cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Audrey Robinson-White	Guest Researcher	LCP	NHLBI
Other: Michael A. Beaven	Deputy Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

Dr. Stephen Baylin, Johns Hopkins School of Medicine, Baltimore; Dr. Thomas Olivecrona, Dept. of Chemistry, Univ. of Umea, Sweden.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Purified microvascular endothelial (MVE) cell preparations from rat and guinea pig fat pad bound diamine oxidase (DAO) in aqueous extracts of rat and human placenta, and in purified preparations of DAO from rat placenta. Scatchard plots indicated the presence of high and low affinity binding sites for DAO. MVE cells also bound purified lipoprotein lipase (LPL). However, bound LPL was not displaced by DAO activity except at high concentrations of DAO. Conversely, bound DAO was only partially displaced by high concentrations of LPL. Binding of both enzymes was blocked by heparin. Each enzyme may thus be bound to two or more populations of sites and one population may bind both enzymes.

Project Description:

Objectives: This project is concerned with the mechanism of clearance of histamine from the circulation after physiological or immunological stimuli. Labeled histamine has been shown to be rapidly removed from the circulation (Klin. Wochenchr 60: 873, 1982; Brit. J. Pharmacol. 49:569,1973) and appears as methylated and deaminated metabolites in all tissues within minutes of injection. When infused through whole animals or isolated organs, histamine was cleared upon a single passage through several vascular beds. We have stated in previous reports (Z01 HL 00631-01 CM: Z01 HL 00631-02 LCP and Z01 HL 00631-03 LCP) that microvascular endothelial (MVE) cells contain high levels of the histamine degrading enzyme histamine methyltransferase (HMT), as well as lower levels of DAO, and that MVE cells thus may be a major site for the inactivation of circulating histamine. The histamine degrading enzyme diamine oxidase is produced in high amounts by the maternal placenta, which results in large increases in enzyme activity in plasma during pregnancy (Acta Physiol. Scand 9:1-107, 1944). In nonpregnant animals, the enzyme is released into the circulation upon injection of heparin (Acta Med. Scand.180:533-536, 1956). Lipoprotein lipase (LPL) activity exhibited analogous behaviour in that it is released into the circulation by heparin. As LPL has been shown to bind to vascular endothelial cells in culture and is displaced from these cells by heparin (J. Biol. Chem. 256,1289,1981) the possibility exists that DAO is bound in a similar manner. This year, we have defined the characteristics of DAO binding to MVE cells and the displacement of bound DAO by heparin and LPL.

Methods Employed: 1) Isolated cell preparations and assay procedures: MVE cells from rat and guinea pig fat pad were prepared as described in project reports for 1980 to 1983. They were assayed for viability and DAO activities by procedures outlined in the same reports. I^{125} LPL was assayed by gamma counting of I^{125} content.

2) Enzyme preparations and extracts. DAO soluble extracts were prepared from rat or human placental extracts and purified by use of sepharose cadaverine affinity columns. LPL and I^{125} LPL were isolated from bovine milk by heparin - sepharose columns (Biochem.J.167,109,1977;B.B.A. 397,294, 1975). DAO activity is expressed as pmoles of histamine deaminated/hr when assayed in the presence of 75 pmoles histamine/ml.

3) DAO and LPL binding studies. For measurement of DAO and LPL binding to MVE cells, cell pellets (1.0 mg, 20 µg protein) were suspended in 0.1 ml phosphate buffered saline (PBS) which contained DAO extract, purified DAO enzyme or purified LPL enzyme (5 to 60 µg/ml) and incubated at 37°C (with DAO) or 22°C (with LPL) for various times. The suspensions were centrifuged (Beckman TJ6) at 500 x g for 3 min, the supernatant fraction was removed and the pellet fraction was resuspended in 0.1 ml PBS (4°C). The cell pellets were sonified and both supernatant and pellet fractions were assayed for DAO activity or for I^{125} LPL content.

4) Displacement of bound DAO activity with heparin or LPL preparations: MVE cells (21 µg protein) were incubated with purified DAO (5,000 pmoles/hr/ml) or DAO extract (100 pmoles/hr/ml) for 1 hr at 37°C. They were recovered by centrifugation (500 x g, 3 min), washed once then incubated in 0.1 ml PBS con-

taining heparin (0.1 to 40 µg/ml) or purified LPL (2.5 to 30 µg/ml). Cells and medium were separated by centrifugation and assayed for DAO as above.

5) Displacement of bound I^{125} LPL with purified DAO: MVE cells (21 µg protein) were incubated at 22°C for 30 min with I^{125} LPL (2.5 to 30 µg/ml) in 0.1 PBS. Cells were recovered, washed once, and resuspended in 0.1 ml PBS containing purified DAO (5,000 pmoles/hr/ml). The suspensions were further incubated at 22°C for 30 min. Cell pellets were assayed for I^{125} LPL and DAO activity.

Major Findings: 1) Binding of DAO extract to MVE cells and displacement by heparin: Preliminary studies with rat or human DAO placental extracts showed binding of DAO to both rat and guinea pig MVE cells. The percentage of DAO activity bound ranged from 17 to 32% with rat and upto 44% with guinea pig MVE cells. Near maximum binding was observed 10 min and appeared constant from 30 to 60 min. DAO activity was displaced (70 to 90%) by 0.05 to 40 µg heparin/ml within 2 min.

2) Binding of purified DAO and LPL preparations to MVE cells. Purified DAO preparations (1,500 pmoles/hr/ml) showed binding at 37°C and to a lesser extent at 4°C. Binding was near maximal at 10 min (16 pmoles/hr/ml) and was maximal by 30-60 min. Scatchard analysis showed at least 2 classes of binding sites; a high affinity site (K_m $10^{10}M^{-1}$) and a low affinity site (K_m $10^{8}M^{-1}$). In addition, rat MVE cells bound 27-33% of LPL added at low enzyme concentrations (12 µg/ml) and less than 25% at high concentrations. However, saturation of LPL binding was not achieved by LPL concentrations up to 60µg/ml.

3) Displacement of DAO activity by LPL and displacement of bound I^{125} LPL by DAO. Bound DAO activity was displaced (22%) only by high concentrations of LPL (>30 µg/ml), and conversely bound I^{125} LPL was displaced only by high concentrations of DAO activity. The lack of displacement of DAO activity or LPL activity may indicate separate binding sites for DAO and LPL on MVE cells although there may be overlap of binding of the two enzymes on each of these sites.

Significance to Biomedical Research and the Program of the Institute: The ability of MVE cells to concentrate DAO activity on the surfaces may, in part, account for the rapid clearance of histamine from the circulation. Small increases (> 5 ng/ml) in plasma histamine concentrations are associated with urticarial and cardiovascular reactions in man and concentrations of 10 to 20 ng/ml are seen during severe anaphylatic shock. The data have clinical significance in that the inactivation of histamine by DAO bound to MVE cells may play a crucial role in minimizing these reactions.

Proposed Course of Project: Future studies will focus on the location and disposition of the second histamine inactivating enzyme (histamine-N-methyltransferase) present in MVE cells. This enzyme activity is located in the cell cytosol, but preliminary evidence indicates that the enzyme activity is secreted to the cell exterior.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL
NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1983, through September 30, 1984

The Laboratory, as usual, consists of two quite differently organized groups. One, the Section on Physiological Chemistry under Dr. John Pisano with 8 full and part-time professionals is concerned with the isolation, identification and function of physiologically important peptides; the report of his Section is appended. The second, consisting of the Sections on Chemical Structure and Nuclear Magnetic Resonance under Dr. Henry Fales and Dr. Robert Highet, respectively, are concerned with isolation, elucidating the structures, and studying the properties of biologically important compounds.

Work in the laboratory was necessarily impeded this year for several months by heavy construction work associated with alterations in the halls. Dust was a major problem, but fortunately no permanent damage was sustained by any precision equipment. The helpful assistance of the construction crew in alerting us to scheduling changes is noted.

Activities in synthesis (a new dimension for the laboratory) have been temporarily delayed with the loss of Dr. Norman Schmuff. He will be replaced by Dr. Steven Miller with similar skills. Dr. Schmuff, although originally involved in synthesis, became proficient in computerized approaches to the literature. This skill has spread rapidly throughout the laboratory and we are increasingly called upon to aid others within the Institutes in this area. Although basically a library skill, it is clear that the maximum benefit can be obtained only when the searching is conducted directly by a scientist.

In mass spectrometry, the ^{252}Cf plasma desorption spectrometer has been fully functional for over one year. It is a highly successful technique, providing molecular weight information on a wide variety of compounds including peptides, dyes and their salts, inorganic complexes, arene oxide metabolites, steroid sugar conjugates, etc. Over 200 compounds have been run so far, and we see no diminution in the demand. The technique has proved particularly easy compared to regular mass spectrometry and many possibilities exist for further improvements. Attempts to study the basic physics of the desorption process are underway by categorizing individual events according to their ion multiplicity. Improvements in resolution will be sought through construction of an ion reflector ("reflectron") system. Because of the success of this instrument, the JEOL photoplate high resolution system acquired from NCI last year has been somewhat neglected. However, the photoplate reader system has been repaired and is now fully functional and a capillary GC has been attached. We anticipate renewed interested in this "old" technique since it is capable of very high sensitivity and has the ability to integrate rapidly changing signals, e.g., in capillary GC.

In nmr spectrometry, the Nicolet 360 continues to provide high quality data with minimum downtime. The two-dimensional NMR method, as predicted last year, accounts for a major portion of its use and the method has been extended to in vivo studies for the first time. Thus the unidirectional flux of creatine phosphate to ATP has been measured in the intact rat leg and head. The saturation transfer NMR method has been found to lead to significant magnetization transfer even with small substrate pools and this technique has been compared with 2D NMR. Certain advantages accrue to each while comparison of both yields even more information.

A study of precision in NMR measurements is basic to all experiments and allows the optimum use of the scarce commodity, time. Where pulse angles are unknown, it has been found that they are best measured separately on a sample with strong resonances. Similarly, Nuclear Overhauser Effects have been measured in the JEOL FX-60 and Nicolet NT-360, and it has been demonstrated that they are accurate only to 5-10% so that structural conclusions from such data must be approached carefully.

Studies of the conformer populations of actinomycin by COSY have revealed possible hydrogen bonded interactions and a new pulse technique (A. Sax, NIAMDD) has been used to provide the complete spectral parameters of a set of fused ring hydrocarbons of interest in arene oxide metabolic processes (D. Jerina, NIADDK). The elegance of the COSY method has been further demonstrated by direct comparison with conventional NMR on an alkaloid, undulatine. One experiment using this method provides all necessary spectra parameters.

In spite of the low sensitivity of this nucleus, ^{15}-NMR has been successfully implemented on the Nicolet NT-360 in a study of the tautomerism of 3,5-dipyrrolidinophenol, and we can foresee many further experiments with this important nucleus.

The relaxation times of ^{7}Li have been studied using the NT-360 in a series of experiments designed to determine its utility as a probe in biochemical studies. Its sensitivity to viscosity and binding to ATP and human platelets were demonstrated at concentrations of 1 mmole or less, augering well for a series of experiments involving ionophores and membrane systems. The nucleus ^{7}Li provides about 1,000 times the sensitivity of ^{13}C.

In X-ray crystallography work continues in spite of the construction downtime of 12 weeks. Various aspects of conformational analysis and their relation to biological activity via interaction with receptor sites have been investigated. The structure of many compounds have been elucidated (see below) and several new computer facilities implemented (automatic reference ordering, multi-font laser printer, etc.). Revision of the X-ray programs as FORTRAN-77 replaces FORTRAN-66 is underway with J. M. Stewart (Univ. of MD).

Insect pheromones have been an ongoing interest of the laboratory, challenging our mass spectral and other techniques in sensitivity. This year we were able to elucidate the structure and synthesize the economically important rice and maize weevil aggregation pheromone (W. Burkholder, Univ. of Wisconsin). The compound, called sitophilure will probably be in commercial production shortly

as its use in the protection of grain supplies is developed. The cause of the unusual phenomenon of "temporary social parasitism" in Bothriomyrmex syrius ants was elucidated as 6-methyl-5-heptene-2-one. The structures of a wide variety of other insect substances were also elucidated (see below).

Natural products from plants are also under investigation, although far less than in past years. An extract from Brunfelsia grandiflora, used in the Amazon as a hallucinogen, has been isolated by following its biological activity (serotonin-like) in mice. It has the simple formula C_6H_7NO and shows unusual spectral features.

In related studies, either alone or in collaboration (see individual reports for identification of collaborators), members of this laboratory have this year: 1. Identified the mandibular secretion of Camponotus thoracicus as 2,4-dimethylhexan-5-olide. 2. Identified ten monoterpenes in heads and gasters of Myrmicaria brunnea. 3. Identified geranyllinalool, a sesquiterpene (possibly γ-cadinene), an unknown sesquiterpene alcohol in Reticulitermes, new species and tetradecyl propionate as the permanent trail pheromone in these termites. 4. Elucidated crystal structures of three morphinanes used as probes of narcotic receptors. 7. Completed x-ray structures of several colchicine analogues. 8. Identified a blue pigment (from a medieval painting) as a complex copper acetate. 9. Identified the structure (NMR) of the dendrobatid alkaloid 239 AB. 10. Showed (NMR) that glucose-6-phosphate and galactose-6-phosphate in contrast to the free sugars or their sulfates anomerize at an appreciable (<0.1 sec^{-1}) rate.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGICAL CHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983, to September 30, 1984

The Section on Physiological Chemistry is primarily concerned with biologically active peptides. Our interests are in the discovery, biosynthesis and the determination of the mode of action of peptides. Two projects continue to be of major interest: the kallikrein-kinin system and the discovery of biologically active peptides.

KALLIKREIN-KININ SYSTEM

Bradykinin is one of the most potent vasodepressor and pain-producing substances known. In blood, bradykinin is formed by kallikrein (an enzyme) acting on the protein substrate, kininogen. Kallikrein also occurs in many exocrine glands, including the kidney. Glandular kallikrein has different physicochemical properties from plasma kallikrein and the two enzymes appear to have totally different roles. In the kidney, the kallikrein-kinin system may have an important function in the regulation of renal tubular electrolyte and water transport. We previously showed that in the rat, kallikrein is highly localized in the distal nephron with the highest concentration occurring in the connecting tubule. We also showed that the site of highest kinin binding was downstream, i.e, in the cortical and medullary collecting ducts. This indicates that collecting ducts are a major site of action of bradykinin.

Action of Bradykinin on the Rat Cortical Collecting Duct. In a collaborative study with Dr. Mark Knepper of the Laboratory of Kidney and Electrolyte Metabolism, NHLBI, we have studied the direct effect of bradykinin on sodium and potassium transport on the isolated perfused rat cortical collecting duct. Sodium, potassium and inulin concentrations were measured in the perfusion fluid, bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. The potential difference across the epithelium was also measured. Arginine vasopressin and/or bradykinin were placed in the bath or in the perfusate. Animals were also pretreated with deoxycorticosterone. Deoxycorticosterone pretreatment of rats for 7-12 days caused a marked increase in sodium absorption and potassium secretion and changed the transepithelial potential difference to lumen negative. There was no fluid transport. Addition of bradykinin to the bath significantly decreased sodium reabsorption without affecting potassium secretion. However, bradykinin did not significantly change the potential nor was there any demonstrable fluid transport. Compared with bradkykinin, vasopressin caused a striking increase in sodium absorption in association with a significant fluid absorption and marked increase in the lumen-negative potential. In addition, vasopressin significantly increased potassium secretion. All effects of vasopressin were reversed when the hormone was removed from the bath. Of greater interest, when bradykinin was added to the bath already containing vasopressin, there was a significant reversible fall in sodium reabsorption and fluid transport with no change in potassium secretion or the potential. These effects were previously observed with bradykinin in the absence of vasopressin but to a lesser extent due to the lower baseline values in the absence of vasopressin.

Addition of bradykinin to the perfusion fluid up to 10^{-6} M was without effect. These data, show for the first time, a direct inhibitory effect of bradykinin on sodium absorption. Furthermore, this occurred with no attendant decrease in potassium secretion, or change in the transepithelial potential, raising the possibility that bradykinin also affects the transport of an anion such as chloride on bicarbonate.

NEW PEPTIDES

Convinced that numerous peptides with profound biological activities await discovery in man, we have an ongoing program on the isolation and characterization of peptides from naturally-occurring rich sources, such as insect and animal venom, and amphibian skin. The rich sources are logical choices because it is highly probable that the structure of the new peptides will be closely related if not identical with peptides normally present in minute quantities in human tissues. The rationale includes: (1) development of suitable micro procedures for the isolation and characterization of peptides, (2) development of biological screening tests (a critical step for the discovery of new peptides), (3) production of antibodies to the new peptides, and (4) use of the antibodies for the detection of cross-reacting peptides in man.

The amino acid sequences of two closely related peptides from Gila monster (Heloderma suspectum) venom have been determined. Helospectin I is a 38 residue peptide and helospectin II is a 37 residue peptide identical to helospectin I except that it lacks serine^{38}. Helospectins are pancreatic secretagogues with structures similar to vasoactive intestinal peptide and other members of the glucagon superfamily (glucagon, secretin, somatoliberin, gastric inhibitory peptide, PHI). Since members of the glucagon superfamily have different biological actions, it is possible that helospectin is more closely related to a mammalian peptide awaiting discovery. The amino acid sequences of five structurally related peptides from bumble bee (Megabombus pennsylvanicus) venom have been determined. The 17-residue peptides, which we have named bombolitin I-V, lyse erythrocytes and lysosomes, release histamine from mast cells and stimulate phospholipase A_2 from difference sources. While the bombolitins represent a unique structural class of peptides, they have biological properties very similar to other structurally unique peptides from insect venoms: melittin from honey bee venom, mastoparan from wasp venom, and crabrolin, a peptide we recently isolated from European hornet venom. This unusual circumstance-peptides with different amino acid sequences having similar biological properties--may be a manifestation of their amphiphilic nature, a structural property these peptides have in common. The amino acid sequences of three peptides from the skin of Rana pipiens have been determined. The structurally similar 24-residue peptides lyse erythocytes and release histamine from mast cells with a potency at least 10 times higher than the bombolitins or crabrolin.

Several peptides have been partially purified from the venom of the snake Oxyuranus scutellatus which interact specifically and competively with calcium binding channels of the dihydropyridine category.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01002-11 CH

PERIOD COVERED

October 1, 1983, to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear Magnetic Resonance Natural Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Edward A. Sokoloski Chemist CH NHLBI

COOPERATING UNITS (if any)

J. Costa, M CN N

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Data on Lithium-7 Nuclear Magnetic Resonance spin-lattice relaxation times in several systems, including human platelets, have been compiled. The nucleus possesses the necessary nuclear magnetic resonance characteristics, such as sensitivity, to be a promising probe for biological systems. Binding to adenosine triphosphate (ATP) and displacement by calcium ion is mirrored by a significant change in the spin-lattice relaxation time of the lithium.

357

Objective

Nuclear magnetic resonance investigations of proton and carbon phosphorus have provided a wealth of information on structure and interactions of biologically important materials. In addition, less common nuclei not found in biological systems but which can be introduced as selective probes have proved useful additions to spectral investigations. Our initial foray into this type of research with the use of fluorodopamine in pig nerve microsacs proved informative. We have, therefore, decided to undertake a feasibility study of lithium-7 as a nuclear magnetic resonance probe in biological systems. The lithium-7 is 1,000 times more sensitive to the NMR experiments than carbon-13. The spectra should be quite simple when the ion is uncoordinated or not strongly bound, but if strongly bound, should provide interesting possibilities for obtaining information about dynamic parameters by virtue of its nuclear quadrupole moment.

Methods Employed

The Nicolet-360 nuclear magnetic resonance spectrometer located in the Laboratory of Chemistry was operated at 140.816 MHz. Samples of lithium chloride were prepared in deionized distilled water with varied amounts of deuterium oxide added to the solution for spectrometer lock and to investigate the mechanism of relaxation processes. Solutions were also made containing lithium chloride and varied concentrations of 1) sucrose, 2) adenosine triphosphate (ATP), 3) adenosine triphosphate and Ca. 4) human blood platelets which had been incubated, washed and resuspended. Measurements were made to determine the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). Temperature studies were also run to determine if the mobility of the lithium ion or complexes that may form would alter the values of the relaxation times to any significant extent.

The spin-lattice relaxation times were determined using a standard inversion-recovery sequence and line fitting the data with a three-parameter exponential line fitting program. The half-band width at half height was used as an approximation for the spin-spin relaxation time. All measurements were made a minimum of 3 times on the same sample, and new preparations of similiar samples were examined several times. This was necessary because of the well-known sensitivity of the spin-lattice relaxation times to impurities, especially paramagnetic ions. The reproducibility of our measurements was found to be better than the often quoted 10% limit of accuracy accepted for this type of measurements.

Major finding

1) The average spin-lattice relaxation times obtained for various solutions, all of which contained 10 mM lithium ion, are summarized in the following tables.

Water Solution	Deuterium Oxide Solution
18.1 secs	28.8 secs

Sucrose	
%	Relaxation Times (secs)
20 "	8.5
46	2.9
60	1.9

Adenosine Triphosphate	
ATP Conc mM	Relaxation Time
1 m	10.7
2	8.4
3	7.6
5	5.6
7	4.9
10	4.5
20	3.3

Adenosine Triphosphate with Calcium

ATP/Ca	Relaxation Times
5:1	3.93
1:1	9.5
1:5	17.1

Human Platelets
7.8 secs

2) Examination of the relaxation times leads to several conclusions:

a) the inter-molecular dipole-dipole mechanism is of considerable importance demonstrated by the effect of deuterium substitution in the solvent.

b) the relaxation time is quite sensitive to viscosity as demonstrated by the sucrose experiment. If the lithium were to be incorporated into biological material with a high viscosity, the lithium could be used as an effective probe.

c) the relaxation time also mirrors the binding of the lithium to adenosine triphosphate and, because of the higher affinity of ATP for calcium, can be displaced by the calcium ion. The displacement results in the relaxation time increasing to the value of free ion in water. This opens interesting possibilities for ionophore and membrane transport studies.

3) The sensitivity of lithium NMR is quite adequate for many types of biological experiments. Concentration of 1 millimole or less seem to be sufficient to obtain good signal to noise ratios, and in time periods suitable for sensitive biological materials.

Proposed Course

Continuation of this project is proposed. NMR observation of the lithium in the presence of ionophores and in membrane systems are possibilities.

Publications

1. Waddell, T. G., Osborne, C. B., Collison, R., Levine, M. J., Cross, M. C. Silverton, J. V., Fales, H. M., and Sokoloski, E. A. Erigerol, a new labdane diterpene from Erigeron philadelphicus. J. Org. Chem., 48: 4450-4453, 1983.

2. Kovac, P., Sokoloski, E. A., and Glaudemans, C. P. J. Synthesis and characterization of methyl 6-O-α- and -β-D-galactopyranosyl-β-D-Galactopyranoside. Carbohydrate Research 128: 101-109, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-01003-12 CH

PERIOD COVERED

October 1, 1983, to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure of Natural Products Using Instrumental Methods

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) Name, title, laboratory, and institute affiliation

PI:	H. M. Fales	Chief, Laboratory of Chemistry	CH	NHLBI
OTHER:	N. R. Schmuff	Staff Fellow	CH	NHLBI
	L. Pannell	(Guest Worker, DSIR, New Zealand)		

COOPERATING UNITS (if any)

Walter Reed (J. Scovill), NCI (O. Gansow, M. Sohn), Univ. of MD. (P. Callery), DSIR, New Zealand (D. Russell), Laboratory of Biochemistry, NHLBI (L. Tsai), Univ. of GA. (M. S. Blum), U. S. Army (T. Krishnamurthy), Univ. of Wisc. (W. Burkholder)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A wide variety of compounds have been examined by ^{252}Cf plasma desorption mass spectrometery including cationic and anionic dyes, inorganic anti-malarials, radioactive nuclide binding ligands, ^{15}N-glutamine, toxic terpenoid glycosides, nonapeptides, organoselenium compounds, ruthenium bipyridils, tetraalkylammonium salts, tetraphenyl porphryin complexes, cyclopeptides, and ion correlation experiments have been initiated. Several new compounds have been discovered in Ptiloglossa amazonen and the cowpea weevil. The structure of the maize and granary weevil has been elucidated as R*S*-5-hydroxy-4-methyl-3-hexanone and it has been synthesized.

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1. The JEOL 01SG-2 photoplate mass spectrometer has been studied in regard to its photoplate reading program. The program parameters have all been varied with consequences that allow elucidation of its construction and optimum use.

2. The ^{252}Cf plasma desorption spectrometer is now fully functional. Spectra have been run as follows:

a. A study of 30 dyestuffs has shown that all but two give adequate molecular weight information in either the positive or negative modes. Fragmentation, when it occurs, is logical and follows thermal principles.

b. A series of inorganic complexes developed by the Army as antimalarial agents (J. Scovill, WRAIR) have been studied and their structures correlated with their spectra. This group also provided a series of bruceine quasinoids of high molecular weight that showed good quasimolecular ions.

c. A series of ligands used with radioactive nuclides to bombard tumors in vivo (O. Gansow, NCI) exhibited good quasimolecular ions and were useful in confirming their structures.

d. Glutamine is so thermally sensitive, converting to pyrollidone carboxylic acid, that its ^{15}N analogs cannot be usefully studied with ordinary spectrometers. Intense M+H and M+Na ions were obtained using ^{252}Cf (P. Callery, U. of MD)

e. A series of terpenoid glycosides gave excellent molecular ions confirming their structures (D. Russell, DSIR, New Zealand). These compounds are apparently highly toxic to sheep in that area.

f. A nonapeptide related to LHRH has given an intense quasimolecular ion and fragment ions, confirming that it has undergone anisoylation at a glutamic residue (J. Rivier, M. Sporn, NCI).

g. A series of organoselenium uridine analogs has yielded successfully to the technique. Other methods (EI, CI, etc.) had uniformly failed (L. Tsai, NHLBI).

h. A series of ruthenium bipyridyl and other tetraalkylammonium salts have been examined as high mass standards for use in the ^{252}Cf technique. Their narrow lines, due to absence of metastables, renders them especially suitable for this purpose.

i. A series of tetraphenylporphyrin salts of various metals of interest in tumor therapy (M. Sohn, NCI) yielded excellent quasimolecular ions, and it seems clear that porphyrins in general will work well with the system in the positive ion mode.

j. A series of biologically active cyclopeptides related to the tricothecins have provided ions showing that a series of samples thought to be distinctly different, are in fact basically the same (T. Krishnamurthy,

U. S. Army, Edgewood Arsenal). Their biological activity differences must be ascribed to trace impurities.

k. A series of experiments classifying the individual bombardment events in ^{252}Cf have revealed that many events yield ions that appear to be correlated in a straightforward fashion, i.e, $M+Na^+$ and Na^+, while others bear no such relation. The technique has great potential for reducing chemical noise in the spectra.

3. Several series of insect chemistry studies have been completed or are under study: a) Ptiloglossa amazonen larval secretions contain an interesting substance of molecular weight 166 that may be a new pyrone. b) The economically important cowpea weevil pheromone, m.wt. 154, is still under study, but probably includes a keto function. c) The structure of the important maize and rice weevil aggregation pheromone has been elucidated as R*,S*-5-hydroxy-4-methyl-3-hexanone. Both this and its R*R* isomer have been synthesized and found to be fully active in assays (W. Burkholder, Univ. of Wisconsin). Commercial utilization of the substance is expected soon. d) Identification of 40 substances present in the stings of 4 species of honeybee, related to the so-called killer bee, has been completed and their differences and similarities discussed (M. Blum, Univ. of Georgia).

PUBLICATIONS

1. Blum, M. S., Jones, T. H., Overall, W. L., Fales, H. M., Schmidt, J. O. and Blum, N. A. Exocrine chemistry of the monotypic ant genus Giganteops. Comp. Biochem. Physiol. 75B: 15-16, 1983.

2. Jones, T. H. and Fales, H. M. E-6-(1-Pentenyl)-2H-pyran-2-one from carpenter ants (Camponotus spp.). Tetrahedron Lett. 24, 5439-5440-4453, 1983.

3. Waddell, T. G., Osborne, C. B., Collison, R., Levine, M. J., Cross, M. C. Silverton, J. V., Fales, H. M., and Sokoloski, E. A. Erigerol, a new labdane diterpene from Erigeron philadelphicus. J. Org. Chem., 48: 4450-4453, 1983.

4. Bian, Z., Fales, H. M., Blum, M. S., Jones, T. H., Rinderer, T. E. and Howard, D. F. Chemistry of cephalic secretion of fire bee Trigona (Oxytrigona)tataira. J. Chem. Ecol., 10: 451-461, 1984.

5. Schmuff, N. R., Phillips, J. K., Burkholder, W. E., Fales, H. M., Chen, C.-W., Roller, P. R., and Ma, M. The chemical identification of the rice weevil and maize weevil aggregation. Tetrahedron Lett. 25: 1533-1534, 1984.

6. Fales, H. M., Blum, M. S., Bian, Z., Jones, T. H. and Don. A. W. Volatile compounds from Ponerine ants in the genus Mesoponera. J. Chem. Ecol. 10: 651-665, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01004-13 CH

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization of Natural Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. A. Lloyd	Research Chemist	CH NHLBI
Others:	N. Schmuff	Research Chemist	CH NHLBI
	M. Goldman	Research Chemist	CH NHLBI

COOPERATING UNITS (if any)
Howard Univ. (G. Kapadia), Meharry Medical College (S. Evans), Tel Aviv University (A. Hefetz), University of Georgia (M. S. Blum), and Universite Pierre et Marie Curie (J. L. Clement)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Structure determination of physiologically active compounds of plant or animal origin. Techniques used include chemical degradation and synthesis, mass spectrometry, infra-red, UV and NMR spectroscopy, gas and liquid chromatography.

PHS 6040 (Rev 1/84)

GPO 904-917

1. Physiologically active plant products

   a) the screening of carcinogenic plant extracts was continued (with Govind Kapadia, Ph.D., Howard University). Mass spectrometric data and interpretation were provided.

   b) isolation of a bioactive material from Brunfelsia grandiflora (with Mark Goldman, Ph.D. CH/NHLBI). Water extracts of this plant are used by Amazon natives to induce analgesic and hallucinogenic effects. Over the past 100 years the presence of various coumarins and alkaloids has been reported in Brunfelsia species, but none was found responsible for its striking physiological action. Using intraperitoneal administration of aqueous solutions in mice to follow the isolation, we were able to obtain a bioactive fraction by ion exchange chromatography and isolate a semi-crystalline material. It is a nitrogen containing compound, of apparent molecular weight 109 (C_6H_7NO). Its mass spectral, UV and NMR properties do not correspond to any known compound. Other modes of isolation, such as preparative liquid chromatography, will be explored to obtain sufficient material for a full structure determination.

2. Synthetic potential dopaminergic and hypotensive drugs (with Stanley Evans, Ph.D. Merharry Medical College). This project was continued. Mass spectrometric studies were conducted here.

3. Insect Pheromones. Determination of structure of unknowns by GC-MS, NMR, chemical degradation or synthesis (in collaboration with Abraham Hefetz, Ph.D., Tel Aviv University, Israel; Dr. Jean Luc Clement, Universite Pierre et Marie Curie, Paris, France; Murray Blum, Ph.D, University of Georgia; and Norman Schmuff, Ph.D., CH/NHLBI.

   a) Investigation of the mandibular secretion of the ant Camponotus thoracicus was completed. The structure of the unknown component, the δ-lactone, was determined as 2,4-dimethylhexan-5-olide.

   b) Study of the anal secretions of workers and female alates of the ant Bothriomyrmex syrius was completed. It explains how the Bothriomyrmex ants can be "temporary social parasites" of another genus, the Tapinoma. Although the workers' secretion consists of mono-unsaturated C_{12} acid (3-Z-dodecenoic acid), the females produce no acid but 6-methyl-5-hepten-2-one. This ketone is the main defensive pheromone of the Tapinoma ants.

   c) Volatiles of Myrmicaria brunnea. Heads and gasters extracts were examined by GC-MS. Ten monoterpenes were isolated and identified; α-pinene, β-pinene, myrcene, α-phellandrene, p-cymene, α-terpinene, cineole, limonene, γ-terpinene and terpinolene.

d. Reticulitermes. The secretions of two termite species was investigated.

(1) Reticulitermes, new species. The main component of this termite secretion was shown to be geranyllinalool ($C_{20}H_{34}O$, MW 290). Trace amounts of a sesquiterpene (MW 204, possibly -cadinene) and a sesquiterpene alcohol (MW 224, structure unknown) were also found.

(2) Reticulitermes sp., a southeastern termite. The structure of the component of the sternal glands was determined by GC-MS and by synthesis. The compound tetradecyl propionate is the permanent trail pheromone of these termites.

Publication

1. Lloyd, H. A., Schmuff, N. R., and Hefetz, A. Chemistry of the male mandibular gland secretion of the carpenter ant, Camponotus thoracicus Fellah Emery. Comp. Biochem. Physiol, 78B, 675, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01005-13 CH

PERIOD COVERED

October 1, 1983, to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

X-Ray Structural R&D for Physiologically Important Molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

J. V. Silverton Research Chemist CH NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The X-ray work of the Laboratory of Chemistry, NHLBI concerns solid state investigations of biologically interesting compounds. Several aspects of drug action have been investigated: opiates, colchinoids and actinomycin. Progress has been made in extending the range of X-ray direct methods and in obtaining adequate data from very small compounds to allow microanalysis by crystallographic techniques.

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Project Description

Objectives: The aim of the work is to use X-ray crystallography to increase our knowledge of physiological processes and to assist other forms of chemical investigation. The techniques are used when they are the methods of choice or necessity. Another objective is to extend the range of X-ray techniques by theoretical and technical developments. As far as possible it is intended to correlate solid state results with those obtained by other physical methods.

Continuous research in the laboratory was interrupted twice this year. First, the diffractometric equipment was moved from Rm. 7N316 to 7N307 and second, a serious dust problem required the shut down of the precision mechanical equipment for about 12 weeks. Necessary vacation of office and laboratory space for periods of several hours to allow construction work, severely impeded smooth operations. Unexplained disappearance of critical notes and papers also caused problems.

Major aspects of research and their significance.

1. Probes of narcotic receptors (with Drs. K. Rice and T. Burke, NIADDK). This project is about two-thirds complete now and involved three crystal structures to establish conformations and confirm structure and a fourth to establish an absolute configuration.

2. Collaborative work in chemical synthesis (with Drs V. Marquez, NCI; D. Stec, Bureau of Biologics and E. May, NIADDK). The crystal structures of three reaction intermediates have been determined (not always, unfortunately, as expected by makers of the compounds). However, accurate knowledge of chemical composition, even if not as expected, can prevent the waste of considerable time. Further projects with the above scientists are in progress.

3. Collaboration in solid state-NMR experiments (with Dr. D. Torchia, NIADDK). This project involves a fundamentally important amino-acid, phenylalanine. The crystal structure of racemic p-fluoro-perdeuterophenylalanine has been determined and the unusual molecular packing is being correlated with the solid state NMR results. The results also shed light on the remarkable fact that the crystal structure of phenylalanine has never been determined (and, in the opinion of JVS, is not likely to be),

4. Colchicine (with Dr. A. Brossi, NIADDK). The assessment of the results of this project has been completed and a full paper has been submitted to J. Amer. Chem. Soc.

5. Computer aspects. The availability of new computational facilities and the retirement of others has necessitated a certain amount of work.

a) FORTRAN-66 is about to be replaced, after a very long and productive life, by FORTRAN-77. It has been necessary to update many programs to the new standards and a collaborative effort to implement the RATFOR program system XTAL (with Dr. J. M. Stewart, University of Maryland) is in progress.

b) The Command Procedure facility of the IBM computer has been utilized to produce an automatic reference ordering facility. This procedure has been used by at least two other scientists in the laboratory and will be made available to the general NIH scientific community.

c) Experience has been gained and detection of programming defects has now allowed us to confidently use the multi-font laser printer capabilities of DCRT.

d) New computer graphics facilities for the Laboratory of Chemistry have been selected and ordered. It is anticipated that a certain amount of programming efforts will be necessary next year.

6. Guest workers in the X-ray laboratory:

Dr. W. H. De Camp (FDA); crystal structure of reaction intermediates.

Dr. V. M. Orna (College of New Rochelle): investigation of medieval pigments by single crystal diffractometry. Dr. Orna is a professor of analytical chemistry and it was possible to identify a blue pigment, which had resisted other techniques, as an unusual complex copper acetate. It is anticipated that this collaboration will continue as Dr. Orna isolates further pigments.

PUBLICATIONS

1. Burke T. R., Jacobson, A. E., Rice, K. C., and Silverton, J. V. Probes for narcotic receptor mediated phenomena 4. Synthesis of (+)-2,3,4,5, 6,6a-hexahydro-3-methyl-8-hydroxy-1H- (4,11b)-methanobenzofuro(3,2d)-azocine, an oxide bridged 5-(m-hydroxyphenyl)-morphan. J. Org. Chem. 49, 1051-1056, 1984.

2. Marquez, V. E., Rao, K. V. B., Silverton, J. V., and Kelley, J. A. A ring expansion approach to 1,3-diazepin-2-one nucleosides. J. Org. Chem. 49, 912-919, 1984.

3. Waddell, T. G., Osborne, C. B., Collison, R., Levine, M. J. Cross, M. C. Silverton, J. V., Fales, H. M., and Sokoloski, E. A. Erigerol, a new labdane diterpene from Erigeron philadelphicus. J. Org. Chem. 48, 4450-4453.

4. Burke, T. R., Jacobson, A. E., Rice K. C., and Silverton, J. V. Probes for narcotic receptor mediated phenomena 6. Synthesis of (+-)-(1 , 4a , 9a)-1,3,4,9a-tetrahydro-2-methyl-2H-(1,4a) - Propanobenzofuro [2,3c]pyridi-8-ol, an oxide-bridged 5-(3-hydroxyphenyl)morphan. J. Org. Chem. (1984) in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NATIONAL INSTITUTES OF HEALTH

INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01006-13 CH

October 1, 1983, through September 30, 1984

PERIOD COVERED BY THIS PROJECT REPORT

The Characterization of Natural Materials

TITLE OF PROJECT

PRINCIPAL INVESTIGATOR(S) AND other professional personnel (below the Principal Investigator) Name, title, laboratory, branch, institute

R. J. Highet Research Chemist CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. Structural Characterization by NMR Spectroscopy. Conventional and two dimensional studies of the alkaloid undulatine have been compared.

2. Nitrogen-15 NMR Studies: N-15 Spectra of the tautomers of 3,5-dipyrrolidinophenol have revealed an unsuspected component.

3. Poison Frog Toxins: A structure has been assigned to the alkaloid 239AB.

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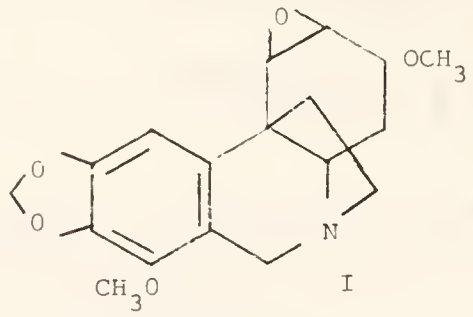
1. Structural Characterization by NMR Spectroscopy.

The alkaloid undulatine, I, has been chosen for examination by conventional



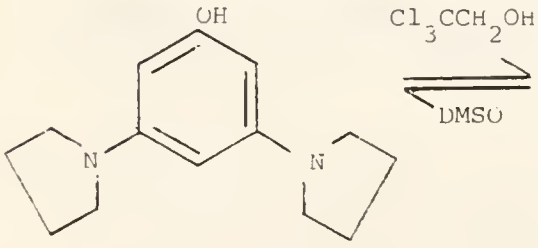
I

and two-dimensional nmr techniques to allow their comparison. Extensive homonuclear decoupling experiments have been compared with two-dimensional correlated spectroscopy (COSY). The two-dimensional technique allows internuclear couplings to be displayed from a single experiment and in a single plot, with superior resolution to that of the conventional experiment. The studies confirm all aspects of the structure shown by early degradative studies.

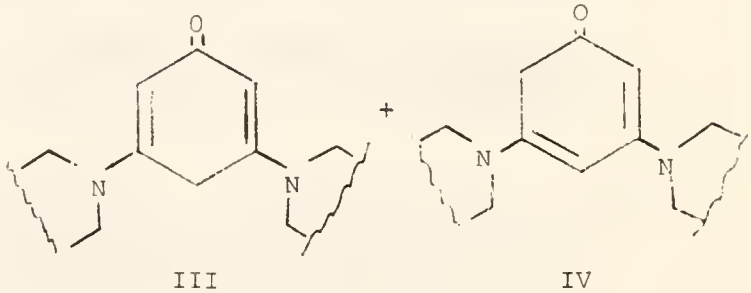
Two-dimensional chemical shift correlation mapping (CSCM) of this alkaloid has to date been unsuccessful. It is suspected that this is an idiosyncrasy of the proton chemical shifts.

2. Nitrogen-15 NMR Studies.

The use of N-15 spectra has been initiated by the study of the tautomerism and reactions of 3,5-dipyrrolidinophenol, II. As anticipated, the poor sensitivity of this nucleus has led to substantial experimental difficulty. However, spectra from molar concentrations showed that the symmetrical dienone, III, is present in



II

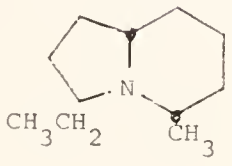


III IV

solutions of II in hydrogen-bonding solvents, as well as the major tautomer, IV, the 2,4 dienone previously observed.

Poison Frog Toxins.

In a collaborative study with Dr. John Daly (NIADDK), the dendrobatid alkaloid 239AB has been shown to have the structure V. Although the stereochemistry has not been firmly established, the carbon-13 chemical shifts imply that shown.



V

Publications:

1. Pohl, L. R., Schulick, R. D., Highet, R. J., and George. J. W.: Reductive-oxygenation Mechanism of Metabolism of Carbon Tetrachloride to Phosgene by Cytochrome P-450. Mol. Pharmacol, 25: 318-321, 1983.

2. Highet, R. J.: The Characterization of Trisubstituted Double Bonds Using $^{3}J[CH]$-Modulated Spectra on Simple NMR Spectrometers. Org. Magn. Reson., 22: 136-139, 1984.

3. Tokuyama, T., Daly, J. W., and Highet, R. J.: Pumiliotoxins: Magnetic Resonance Spectral Assignments and Structural Definition of Pumiliotoxins A and B and Related Allopumilio-toxins. Tetrahedron, 40: 1183-1190, 1984.

4. Jones, T. H., Highet, R. J., Fales, H. M., and Blum, M.: (5z,9z)-3-Alkyl-5-methylindolizidines from Solenopsis (Diplorhoptrum) Species. J. Chem. Ecol., 10: 1233-1249, 1984.

5. Daly, J. W., Highet, R. J., and Myers, C. W.: Occurrence of So-called Dendrobatid Alkaloids in Phyllogenetically Unrelated Anurans of Brazil (Bufonidae), Malagasy (Mantellidae), and Australia (Leptodactylidae), Toxicon, in press.

6. Highet, R. J. and Wheeler, J. W.: The study of Alkaloid Structures by Spectral Means, in "The Alkaloids" (A. Brossi, ed.) Vol. 25, in press.

7. Ferretti, J. A., Highet, R. J., Pohl, L. R., Monks, T. J., and Hinson, J. A: Two-Dimensional J-Resolved Nuclear Magnetic Resonance Spectral Study of Two Bromobenzene Glutathion Conjugates. Environmental Health Perspectives, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01012-11 CH

PERIOD COVERED
October 1, 1983, to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Peptide Biochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

PI:	J. J. Pisano	Head, Section on Physiological Chemistry	CH NHLBI
OTHERS:	A. Argiolas	Visiting Associate	CH NHLBI
	M. Goldman	Staff Fellow	CH NHLBI
	P. Herring	Chemist	CH NHLBI
	P. Highet	Chemist	CH NHLBI
	R. Horikawa	Visiting Fellow	CH NHLBI
	D. Parker	Chemist	CH NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
5.4	5.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The amino acid sequences of two closely related peptides from Gila monster (Heloderma suspectum) venom have been determined. Helospectin I is a 38 residue peptide and helospectin II is a 37 residue peptide identical to helospectin I except that it lacks serine[38]. Helospectins are pancreatic secretagogues with structures similar to vasoactive intestinal peptide and other members of the glucagon superfamily (glucagon, secretin, somatoliberin, gastric inhibitory peptide, PHI). Since members of the glucagon superfamily have different biological actions, it is possible that helospectin is more closely related to a mammalian peptide awaiting discovery. The amino acid sequences of five structurally related peptides from bumble bee (Megabombus pennsylvanicus) venom have been determined. The 17-residue peptides, which we have named bombolitin I-V, lyse erythrocytes and lysosomes, release histamine from mast cells and stimulate phospholipase A_2 from difference sources. While the bombolitins represent a unique structural class of peptides, they have biological properties very similar to other structurally unique peptides from insect venoms: melittin from honey bee venom, mastoparan from wasp venom, and crabrolin, a peptide we recently isolated from European hornet venom. This unusual circumstance-peptides with different amino acid sequences having similar biological properties--may be a manifestation of their amphiphilic nature, a structural property these peptides have in common. The amino acid sequences of three peptides from the skin of Rana pipiens have been determined. The structurally similar 24-residue peptides lyse erythocytes and release histamine from mast cells with a potency at least 10 times higher than the bombolitins or crabrolin. Several peptides have been partially purified from the venom of the snake oxyuranus scutellatus which interact specifically and competively with calcium binding channels of the dihydropyridine category. Finally, all the common dansyl amino acids have been separated in a single high performance liquid chromatographic run.

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Major Findings:

Helospectins: We previously reported that Gila monster venom contains a peptide(s) which stimulates amylase secretion from dispersed guinea pig pancreatic acini by a mechanism involving activation of adenylate cyclase. Two active peptides have been purified from the venom by high performance liquid chromatography and their amino acid sequences determined by automated Edman degradation using the Beckman 890 sequencer. The sequences were deduced from end-group and sequencer analyses of the intact peptides and their tryptic fragments. We have named the peptides helospectins I and II. The structure of helospectin I is: His-Ser-Asp-Ala-Thr-Phe-Thr-Ala-Glu-Tyr-Ser-Lys-Leu-Leu-Ala-Lys-Leu-Ala-Leu-Gln-Lys-Tyr-Leu-Glu-Ser-Ile-Leu-Gly-Ser-Ser-Thr-Ser-Pro-Arg-Pro-Pro-Ser-Ser. Helospectin II has the identical structure except that it lacks the last residue, serine[38]. Helospectins are pancreatic secretagogues with structures and bioactivities similar to vasoactive intestinal peptide (VIP). The sequences of helospectins clearly places them in the glucagon superfamily. By dividing the number of identical residues by the total number in each peptide in the family, one obtains: VIP 15/28, PHI, 14/27, somatoliberin 11/44, glucagon 10/29 and gastrointestinal peptide 7/42. Since members of the glucagon superfamily have different functions, helospectins may be more closely related to a hormone awaiting discovery in man.

Crabrolin and Mastoparan C: We previously reported amino acid sequences of two peptides we discovered in the venom of the European hornet, Vespa crabro. We named the peptides mastoparan C and peptide 2. We have further characterized peptide 2 which we have named crabrolin. The tridecapeptide releases histamine from rat peritoneal mast cells with a threshold of 2.5 µg/ml (0.8 µM). Crabrolin also facilitates the action of purified phospholipase A_2 from different sources, but it is not quite as active as mastoparan. It was clearly less active than mastoparan in lysing erythrocytes, and it did not release amylase from dispersed guinea pig pancreatic acini. Given its unique sequence, the principal effect of crabrolin may be neither mast cell degranulation nor phospholipase facilitation but a yet undiscovered action.

Bombolitins:

Five heptadecapeptides rich in hydrophobic amino acids have been discovered in the venom of the bumble bee Magabombus pennsylvanicus and the amino acid sequence determined. We named them bombolitin I (Ile-Lys-Ile-Thr-Thr-Met-Leu-Ala-Lys-Leu-Gly-Lys-Val-Leu-Ala-His-$ValNH_2$), bombolitin II (Ser-Lys-Ile-Thr-Asp-Ile-Leu-Ala-Lys-Leu-Gly-Lys-Val-Leu-Ala-His-$ValNH_2$), bombolitin III (Ile-Lys-Ile-Met-Asp-Ile-Leu-Ala-Lys-Leu-Gly-Lys-Val-Leu-Ala-His-$ValNH_2$), bombolitin IV (Ile-Asn-Ile-Lys-Asp-Ile-Leu-Ala-Lys-Leu-Val-Lys-Val-Leu-Gly-His-$ValNH_2$) and bombolitin V (Ile-Asn-Val-Leu-Gly-Ile-Leu-Gly-Leu-Leu-Gly-Lys-Ala-Leu-Ser-His-$LeuNH_2$). Bombolitins are structurally and functionally very similar. They lyse erythrocytes, release histamine from rat peritoneal mast cells and stimulate phospholipase A_2 from different sources. The threshold dose is 0.5-2.5 µg/ml depending on the peptide and the bioassay. Most interesting is bombolitin V which is as potent as the well-known melittin of bee venom in lysing guinea pig erythrocytes (ED_{50} = 0.7 µg/ml = 4 x 10^{-7} M) and is 5 times more potent than mastoparan in causing mast cell degranulation making it one of the most potent degranulating peptides discovered so far (ED_{50} = 2 µg/ml = 1.2 x 10^{6} M). The bombolitins represent a unique structural

class of peptides but they have the same biological properties as melittin, mastoparan, and crabrolin, peptides from bee, wasp, yellow jacket and hornet venoms. This unusual circumstance--peptides with different amino acid sequences having the same biological properties--may be a manifestion of their amphipathic nature, a structural property these peptides have in common.

P19: A sixth peptide, P19, has been discovered in the venom of M. pennsylvanicus. It was isolated (0.3 nmol/sac compared with 6-23 nmol for the bombolitins) by high performance liquid chromatography and its amino acid sequence determined by analysis of the natural peptide and typtic fragments using manual methods (DABITC-PITC-Edman). Similar results are obtained by analysis of the reduced and carboxymethylated peptide with the automated gas phase sequencer of Applied Biosystems, Inc., located in Dr. E. Appella's lab. P19 contains 28 amino acid residues and two disulfide bridges. The tentative structure is: Met-Cys-Ile-Cys-Lys-Asn-Gly-Lys-Pro-Leu-pro-Gly-Phe-Ile-Gly-Lys-Ile-Cys-Arg-Lys-Ile-Cys-Met-Med Gly-Thr-HisNH$_2$. Studies are in progress to more firmly establish the sequence. Assignments of the disulfide bridges, C-terminal HisNH$_2$, and residues 10 and 14 are the major concerns. It appears that P19 is one of the most potent naturally-occurring releasers of histamine from rat peritoneal mast cells having a threshold level near 0.05 µg/ml. Only one other peptide is known to have comparable activity, the so-called mast cell degranulating peptide of honey bee venom. The structures of the bumble bee and honey bee venom peptides are somewhat related. Both contain two disulfide bridges, several basic and no acidic amino acid residues, and C-terminal amino acid amides. Alignment of the sequences shows that 6 of the 22 residues of the honey bee peptide are identical to the bumble bee peptide. However, the number of peptide bonds between the two disulfide bridges are 12 and 14 in the honey bee and 16 and 18 in the bumble bee peptide.

Peptides in Frog Skin: Analysis of 80% methanol extracts of Rana pipiens skin by high-performance liquid chromatography shows 25 peptide peaks in the molecular weight range 500-3000. Amino acid analyses indicate that 10 of the peptides are related to bradykinin or ranatensin, peptides we previously discovered in these extracts. Three peptides (P23, P24, P25) have been isolated by high-performance liquid chromatography and their amino acid sequences determined. The native peptides and fragments obtained after trypsin digestion were subjected to automated Edman degradation and end-group analysis by the dansyl procedure. The frog peptides have very similar structures. They have 24 amino acids, 4 lysine residues, no acidic amino acids, and they contain one disulfide bridge spanning residues 18 to 24.

Phe-Leu-Pro-Ile-Ile-Ala-Gly-Ile-Ala-Ala-Lys-Val-Phe-Pro-Lys-Ile-Phe-
Cys-Ala-Ile-Ser-Lys-Lys-Cys

Phe-Leu-Pro-Ile-Ile-Ala-Gly-Val-Ala-Ala-Lys-Val-Phe-Pro-Lys-Ile-Phe-
Cys-Ala-Ile-Ser-Lys-Lys-Cys

Phe-Leu-Pro-Ile-Ile-Ala-Ser-Val-Ala-Ala-Lys-Val-Phe-Ser-Lys-Ile-Phe-
Cys-Ala-Ile-Ser-Lys-Lys-Cys

P23 is about as potent a lytic agent (erythrocytes and liposomes) as mastoparan but 10-20 times more potent in causing the release of histamine from mast cells. Preliminary data indicate comparable potency to the bumble bee peptide, P19, mentioned above. Interestingly, P19 was not lytic. It is noteworthy that the frog peptides are basic (like all peptides mentioned), and contain one disulfide bridge.

Analysis of 80% methanol extracts of Xenopus laevis skin by high-performance liquid chromatography shows 19 peptide peaks in the molecular weight range 200-3000. Amino acid analyses indicate that 8 of the peaks are related to xenopsin and caerulein, two peptides previously discovered by others. All 8 peptides cause the release of amylase from dispersed guinea pig pancreatic acini. Of the remaining peptides, only peak 19 was active in this test. It must be purified for further testing.

Peptides Which Affect Calcium Channels. Calcium channels regulate fluxes of calcium between intracellular and extracellular compartment. Several classes of drugs bind to specific sites on calcium channels and pervent transmembrane movement of calcium. Three categories of calcium channel blockers have been considered in setting up screening test based on competitive binding: 1) dihydropyridines (nifedipine, nitrendipine) 2) phenylalkylamines (verapamil, methoxyverapamil) and 3) benzothiazepines (diltizem). [^{3}H]-nitrendipine has been used to label calcium channels and peptides have been sought which compete with [^{3}H]-nitrendipine for binding sites.

Several venoms and toxins have been fractionated by high performance liquid chromatography. Most promising in the venom of the snake, Oxyuranus scutellatus, as it probably contains seven peptides (activity is virtually eliminated by pronase digestion) which displace ^{3}H-nitrendipine from rat brain synaptosomal membranes and guinea pig longitudinal muscle membranes. The peptides had no detectable phospholipase ativity nor did they facilitate phospholipase activity. Also, they did not inhibit [^{3}H]-spiroperidol (ligand of dopamine receptor) and [^{3}H]-flunitrazepam (ligand of benzodiazepine receptor) binding. Further evidence for specific interaction of the peptides with [^{3}H]-nitrendipine binding sites was obtained by kinetic studies which showed that the peptides were competitive inhibitors.

Extracts (80% methanol) of the skins of Xenopus laevis, Rana pipiens and Rana catesbeiana were also tested (as above). Only Rana pipiens appears to have activity.

Endogenous Ligands of the Imipramine Binding Site. Human and rat blood contain substances which inhibit neuronal and platelet uptake of serotonin and binding of imipramine. Fractionation of blood extracts by high performance liquid chromatography gives a partially purified preparation which inhibited [^{3}H]-serotonin uptake and [^{3}H]-imipramine binding to rat cortical synaptosomes and human platelets but did not alter [^{3}H]-serotonin release from synaptosomes. Kinetic analysis showed that [^{3}H]-imipramine binding was competitively inhibited but [^{3}H]-serotonin binding was non-competitively inhibited. More work is required to purify and characterize the active material(s).

Lack of Peptides in Central and South American Frogs. Methanol extracts of skins of 19 species provided by Dr. John Daly, NIADDK have been tested for peptides which stimulate the release of amylase from dispersed guinea pig pancreatic acini. No activity attributable to peptides was found.

Immunoreactive Mastoparan and Granuliberin in Rat Brain and Human Urine. Crude fractions of rat brain and human urine were previously shown to contain immunoreactive mastoparan (see Z01 HL 01012-09 CH). However, studies this year have shown that upon purification of the crude fraction by HPLC, no activity was found which was destroyed by incubation with the protease, subtilisin which inactivates mastoparan. Immunoreativity of the crude extracts has been attributed to interference in the assay caused by multiple components in the extracts which non-specifically inhibited ligand binding.

Immunoreactive granuliberin in rat brain has been fractioned on Sep-Pak C_{18} cartridges. Activity was eluted with 40% acetonitrile containing 0.1% TFA. Increasing amounts of active material caused displacement of labeled granuliberin in a manner which almost paralleled standard granuliberin. Whole brain contains 120 pg/g brain. Hypothalamus contains almost twice as much immunoreactivity (180 µg/g) as cortex, cerebellum and pons. The forebrain has an intermediate level (140 pg/g). More work is required to establish that the immunoreactivity is due to a single entity and that it is a peptide.

Miscellaneous. Since the adenosine previously reported (Z01 Hl 01012-09 CH) in venom of the wasp Polistes annularis is found in high concentration in muscle tissue, we reinvestigated this insect and found that the muscular venom sac, and not the pure venom, is the source of the adenosine.

After testing numerous columns and mobile phases, we have resolved all the common dansyl amino acids under the following conditions: column, Waters 3.9x150 mm (5 spherical C_{18}); eluent A, 30 mM HEPES, 50 mM glycine and 14.3 mM TEA, pH 7.55; eluent B, the same buffer in acetonitrile, 2:3; elution, linear gradient, 20-80% B over 60 min; temp 45° flow, 1.0 ml/min. The last amino acid, di-danyl-tyrosine eluted at 49 min. The time could be shortened to 35 min by using the two step gradient: 20-43% B over 23 min and 43-100% B over the next 17 min. Although the procedure appears to be well suited for the identification of dansyl amino acids at the low picomole level by fluorometric detection, one problem remains--column stability. We are presently unsure if there is a manufacturing defect in column production or if the pH of our eluent, 7.6, causes the silica matrix to break down.

Significant efforts were also expanded on the new Beckman amino acid analyzer Model 6300 to bring it up to specifications. In collaboration with Beckman representatives, new pumps and new tubing were installed and the program was modified to allow accurate analysis down to the 50 pmol level. Programs were also developed to separate tryphophan from ammonia and to analyze all amino acids in an hydrolysate prepared with methane sulfonic acid as well as with hydrochloric acid. The program of the Beckman 890 protein sequencer was also modified to increase the repetitive yield. With the new program, we have raised the repetititve yield from 91% to values as high as 96%.

Proposed Course

1. Study the structure-function relationship of helospectin analogues.

2. Determine the structure of the bumble bee peptide P19.

3. Purify and determine the structures of the biologically-active peptides discovered in Rana pipiens and Xenopus laevis skin.

4. Purify and determine the structures, potency, and mode of action of the peptides in Oxyuranus scutulatus venom which appear to block calcium channels.

5. To characterize immunoreactive ranatensin in brain and pineal gland, and immunoreactive granuliberin in rat brain.

6. To discover new biologically-active peptides in amphibian skin and venoms.

Publications

1. Argiolas, A., and Pisano, J. J. Facilitation of phospholipase A_2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom. J. Biol. Chem. 258, 13697-13702, 1983.

2. Argiolas, A. and Pisano, J. J. Isolation and characterization of two new peptides, mastoparan C and crabrolin from the venom of the European hornet, Vespa crabro. J. Biol. Chem. in press.

3. Parker, D. S., Raufman, J-P., O'Donohue, T. L., Bledsoe, M., Yoshida, H. and Pisano, J. J. Amino acid sequences of helospectins, new members of the glucagon superfamily, found in gila monster venom. J. Biol. Chem. in press.

4. Argiolas, A. and Pisano, J. J. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumble bee Megabombus pennsylvanicus. J. Biol. Chem. in press.

5. Angel, I., Goldman, M. E., Skolnick, P., Pisano, J. J., and Paul, S. M. Characterization of endogenous inhibitors of [^{3}H]-imipramine binding and [^{3}H]-sorotonin uptake from rat serum. In Lal, H. and LaBella, F. S. (eds.) In First International Symposium on Endocoids, A. R. Liss Publishing Co., Inc., in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01016-14 CH

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Clinical Biochemistry of the Kallikrein-Kinin System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. J. Pisano, Head,	Section of Physiological Chemistry	CH NHLBI
Other:	K. Tomita	Visiting Fellow	CH NHLBI
	P. Highet	Chemist	CH NHLBI

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LAB/BRANCH
Laboratory of Chemistry

SECTION
Section of Physiological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To gain a better understanding of the role of the kallikrein-kinin system in the kidney, we have studied the direct effect of bradykinin on sodium and potassium transport on the isolated perfused rat cortical collecting duct. Sodium, potassium and inulin concentrations were measured in the perfusion fluid, bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. The potential difference across the epithelium was also measured. Arginine vasopressin and/or bradykinin were placed in the bath or in the perfusate. Animals were also pretreated with deoxycorticosterone. Tubules were perfused at the rate of 1.5-2.5 nL/min/mm. Deoxycorticosterone pretreatment of rats for 7-12 days caused a marked increase in sodium absorption and potassium secretion and changed the transepithelial P.D. to lumen negative. There was no fluid transport. Addition of bradykinin to the bath significantly decreased sodium reabsorption without affecting potassium secretion. However, bradykinin did not significantly change the P.D. nor was there any demonstrable fluid transport. Compared with bradkykinin, vasopressin caused a striking increase in sodium absorption in association with a significant fluid absorption and marked increase in the lumen-negative P.D. In addition, vasopressin significantly increased potassium secretion. All effects of vasopressin were reversed when the hormone was removed from the bath. Of greater interest, when bradykinin was added to the bath already containing vasopressin, there was a significant reversible fall in sodium reabsorption and fluid transport with no change in potassium secretion or P.D., effects previously observed with bradykinin in the absence of vasopressin (albeit to a lesser extent due to the lower baseline values in the absence of vasopressin.) Addition of bradykinin to the perfusion fluid up to 10^{-6} M was without effect. The combined data show, for the first time, a direct inhibitory effect of bradykinin on sodium reabsorption. In a separate study, kininogen was found in washed homogenates of rabbit kidney.

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Major Findings

Action of Bradykinin on the Rat Cortical Collecting Duct. To gain a better understanding of the role of the kallikrein-kinin system in the kidney, we have studied the direct effect of bradykinin on sodium and potassium transport in the isolated perfused rat cortical collecting duct. Sodium, potassium and inulin concentrations were measured in the perfusion fluid, bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. The potential difference across the epithelium was also measured. Arginine vasopressin (AVP) and/or bradykinin (BK) were placed in the bath or in the perfusate. Animals were also pretreated with deoxycorticosterone (DOC). Tubules were perfused at the rate of 1.5-2.5 nL/min/mm

TABLE 1

Effect of Hormones on Sodium and Potassium Fluxes in the Rat Cortical Collecting Duct

Animal Pretreatment	Hormone* In Bath	Flux (peq/min/mm) Na	K	Potential Diff. mv
NONE	None	0.2	0.1	- 0.8
DOC	None	13.8	- 2.1	- 3.6
	BK	7.2**	- 1.5	- 2.1
DOC	None	12.5	- 5.1	-10.7
	AVP	58.0**	-16.6**	-26.6*
	None	27.7	- 7.3	-13.6
DOC	AVP	51.0	-12.0	-24.5
	AVP&BK	31.2**	- 9.7	-22.1
	AVP	45.3	- 6.2	-21.6

*AVP, 10^{-10} M, BK, 10^{-9} M.
**Significantly different from first value in set

The data in Table I show that deoxycorticosterone pretreatment of rats for 7-12 days caused a marked increase in sodium absorption and potassium secretion and changed the transepithelial P.D. to lumen negative. There was no fluid transport. Addition of bradykinin to the bath significantly decreased sodium reabsorption without affecting potassium secretion. However, bradykinin did not significantly change the P.D. nor was there any demonstrable fluid transport. Compared to bradkykinin, vasopressin caused a striking increase in sodium absorption in association with a significant fluid absorption and marked increase in the lumen-negative P.D. In addition, vasopressin significantly increased K secretion. All effects of vasopressin were reversed when the

hormone was removed from the bath. Of no less interest, when bradykinin was added to the bath already containing vasopressin, there was a significant reversible fall in sodium reabsorption and fluid transport with no change in potassium secretion or P.D., effects previously observed with bradykinin in the absence of vasopressin (albeit to a lesser extent due to the lower baseline values in the absence of vasopressin.) Addition of bradykinin to the perfusion fluid up to 10^{-6} M was without effect. The combined data show for the first time a direct inhibitory effect of bradykinin on sodium reabsorption.

Renal Kininogen

Using human kininogen antibodies, we previously reported cytoplasmic staining in cells of the distal nephron of human kidney. However, in preliminary studies, we found little kininogen in kidney homogenates suggesting that the kidney does not store kininogen and that there is a high turnover rate. We have now focused on the rabbit kidney because kallikrein is highly localized in the cortical connecting segment of the distal nephron and it is important to know if the kallikrien substrate, kininogen, is in the same site. Homogenates of cortex and medulla prepared with phosphate buffered saline were examined. The homogenate was washed five times with buffer (by centrifugation) and the pellet frozen. The thawed pellet was suspended in buffer containing 1% triton X-100 and sonicated. Extracted kininogen as well as the five washes were examined. Kininogen was determined by treatment with trypsin and radioimmunoassay of released kinin. Kininogen was found in the original five washes and in the detergent extract of the washed pellet. A second detergent extraction gave no additional kininogen. When detergent was omitted, half as much kininogen was extracted. The five washes contained decreasing amounts of kininogen with the fifth wash containing about 5% of the first wash. The detergent extract of washed tissue contained about 15% of the total kininogen in the five washes. Finding kininogen in the washed tissue was unexpected because kininogen is a soluble protein. Of no less interest, the washed medulla pellet contained 59 pg kininogen/mg protein and cortex, 21 pg. The amount of kininogen in urine is high relative to the amount of kininogen in kidney. Thus, it appears that there is rapid turnover and little storage of kininogen in the kidney. Since the medulla does not contain kallikrein, this finding suggests that in the kidney, kallikrein from the connecting tubule travels down the nephron to interact with medullary kininogen. Taken with our previous finding of higher specific kinin binding in medulla vs cortex, it seems possible that the medulla is a site of kinin action as well as kinin generation.

Proposed Course

1. Determine the effect of bradykinin on chloride and bicarbonate transport in the isolated perfused rat cortical collecting duct.

2. Determine if bradykinin inhibition of sodium reabsorption is mediated by prostaglandins.

3. Determine the effect of bradykinin on primary cultures of cortical collecting duct cells.

4. Demonstrate the biosynthesis of kininogen in the isolated perfused rat kidney.

Publications:

1. Goldstein, D. J., Ropchak, T. G., Keiser, H. R., Atta, G. S. Argiolas, A., and Pisano, J. J. Bradykinin reverses the effect of opiates in the gut by enhancing acetylcholine release. J. Biol. Chem. 258: 12122-12124, 1983.

2. Marks, E., Alving, B., and Pisano, J. The kallikrein-kinin system in the Brown Norway rat. Thrombosis Research 31: 653-656, 1983.

3. Tomita, K. and Pisano, J. J. Binding of [^{3}H]-Bradykinin in isolated nephron segments of the rabbit. Am. J. Physiol. 246, F732-F737, 1984.

4. Alving, B. M., Niebyl, J. R., Proud, D., Mason, B. L., and Pisano, J. J. Human plasma prekallikrein and high molecular weight kininogen decrease during parturition. Thrombosis Research 34, 473-477, 1984.

In Press

Proud, D., Nakamura, S., Carone, F. A., herring, P. L., Kawamura, M., Inagami, T., and Pisano, J. J. The kallikrein-kinin and renin-angiotensin systems in rat renal lymph. Kidney International.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01018-27 CH

PERIOD COVERED

October 1, 1983, to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of the Kallikrein-Kininogen-Kinin System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

Jack V. Pierce, Research chemist NHLBI, CH

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LAB/BRANCH

Laboratory of Chemistry

SECTION

Physiological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Rat renal kallikrein has been purified by 8 M urea hydroxyapapite chromatography of the immune precipitate produced from a kallikrein fraction of low specific activity and specific sheep antiserum. The antibody, uncontaminated by antigen, passed through the column, while the kallikrein was adsorbed and was later eluted by a linera linear phosphate gradient in 3 M KCl.

Human renal kallikrein and its specific goat antibody are being purified, by the procedure described above for the rat enzyme, from the immune precipitate formed from 1,500 TAMe units (equivalent to 25 mg of pure enzyme) of partially purified kallikrein and nearly two liters of goat antiserum.

In a preliminary experiment, L-arginine attached to agarose via a triazinyl-aminododecyl arm had an acceptable adsorptive capacity for human renal kallikrein, thus showing promise for use in purification and possibly in separating it from its zymogen.

3+3

Objectives:

Purification of glandular kallikreins and prokallikreins and of components of the plasma kinin, clotting, and fibrinolytic systems for purposes of characterization and production of specific antiserums. Preparation of purified specific antibodies and isolation from plant, animal, and other sources of specific inhibitors for human plasma and glandular proteinases (kallikreins, factor XII, plasmin, thrombin, factor XI, elastases, cathepsins, etc.) for biochemical, clinical, and other studies. Preparation of affinity adsorbents with purified antibodies, antigens, enzymes, inhibitors, and various chemical compounds (amino acids, peptides, and dyes) for purification and other purposes, such as devising specific biochemical and radioummunochemical assays. Application of these purified materials, affinity adsorbents, and assay methods to studies of normal and pathological states in man and other primates.

Major Findings:

1. Rat Renal Prokallikrein: Purification of Renal Kallikrein and its Antibody by Hydroxyapatite Chromatography. The antibody and antigen in the immune precipitate described in the 1983 Annual Report (Z01 HL 01018-26 CH) were separated on a hydroxyapatite column in the presence of 8 M urea at about 23°C. Anti-renal kallikrein antibody was found by the Ouchterlony method in the unadsorbed 8 M urea effluent and wash, but has not yet been quantified. After urea had been washed from the column with water, 3 M KCl removed virtually no A_{380} material. However, 3 M KCl/0.01 M NaP_i, pH 6.0, eluted small amounts of both A_{280} material and esterase activity. A_{280} determinations were made on each of the 93 fractions obtained thus far, while esterase activity measurements--using Z-Lys-SBzl as substrate--were done on 9 pools made from the last 69 fractions with significant A_{280} values. Of these fractions, 59 were contributed by a 280-ml linear gradient of 3 M KCl/0.01 M NaP_i, pH 6.0, to 3 M KCl/0.15 M NaP_i, pH 6.0 (24 of the 93 total number of fractions contained the purified antibody and 8 M urea and water washes). The results obtained so far are summarized in the table below:

Pool No.	Effluent Vol., ml	A_{280} units	TAMe units (TU)	% of Total Activity	TU/A_{280}
I	36	0.71	0.086	0.08	0.12
II*	60	3.03	28.7	26.4	9.5
III	48	3.25	60.8	55.9	18.7
IV	48	2.67	10.7	9.8	4.0
V	42	2.98	5.6	5.1	1.9
VI	42	2.81	1.8	1.7	0.64
VII	37	1.79	0.54	0.50	0.30
VIII	32	1.04	0.35	0.32	0.34
IX	34	1.17	0.21	0.18	0.19
TOTAL		19.45	108.7	99.99	5.59

*The gradient was begun after 35 ml of Pool II had been collected.

2. Human Renal Kallikrein. a. Purification of Kallikrein and its Specific Antibody by Immune Precipitation. This was undertaken to obtain both antigen and antibody sufficiently pure for use of the former in radioimmunoassays and of the latter for immobilization at a high degree of substitution on certain synthetic, highly porous particles. About 1,500 TAMe units (25 mg as pure kallikrein with a specific activity of 60 TAMe units/A_{280}) in 182 ml were mixed at equivalence with 1,940 ml of goat antiserum and 1,620 ml of 0.15 M NaCl/0.025 M NaP_i/2 mM EDTA/2 mM EGTA/0.025% NaN_3, pH 7.2 (Buffer A). After incubation at 38°C for 2 h and storage at 0°C for 40 h, the light tan precipitate was collected by centrifugation at 0°C. Following four washes of 550, 265, 90, and 45 ml of Buffer A at 0°C, the precipitate was further washed twice with 45 ml of 0.1 M NH_4OAc, pH 6.8, and once with 20 ml of water containing 0.01% NaN_3. The final supernatant solution had an A_{280} of 0.013 and a conductivity of 1.9 mmho. The final centrifuged precipitate now stored at 0°C has a volume of about 5 ml.

b. The combined active fractions eluted by water from the Phenyl-Sepharose column discussed in the previous annual report, were concentrated by ultrafiltration, diluted with water, and assayed with Z-Lys-SBzl. Again there seemed to be a 30% gain in activity over the starting material. However, it had been diluted in 2 M $(NH_4)_2HPO_4$ for assay. When the concentrated pool was also diluted in this buffer, about the same diminution of esterase activity from the sample diluted in water was observed. On the basis of these results, the concentrated pool now shows only a 78% recovery from the starting fraction.

c. The adsorption of purified human renal kallikrein (21 TU/A_{280}) was tested on columns of immobilized L-arginine and L-lysine. Only the adsorbent in which arginine was separated from the agarose matrix by a triazinylaminododecyl arm showed good binding of renal kallikrein in 0.15 M KCl/0.025 M NaP_i, pH 6.6. Neither arginine attached via a triazinylaminohexyl arm nor lysine attached via a triazinylaminododecyl arm to agarose supports adsorbed any detectable kallikrein.

Proposed Course of Project: Human and Rat Renal Kallikrein and Prokallikrein. Methods will be sought to purify prokallikrein to its maximum specific activity, separate it readily from kallikrein, and especially prevent its activation. For this purpose, various inhibitors will be tested. If one is found, methods of removing a hypothetical activating enzyme can be tried. The Phenyl-Sepharose experiments will be extended in the hope of finding conditions for purifying both kallikrein and prokallikrein, as well as for resolving them. With the same end in mind, other chromatographic methods will be tested. Once we have maximally purified prokallikrein, we will determine the sequence of the activation peptide as well as its location in the proenzyme molecule. The work now in progress on the purification of rat and human renal kallikreins by hydroxyapatite chromatography of their immune precipitates will be continued, and further purification of the kallikreins eluted from the hydroxyapatite columns will be sought.

Publications

none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 01027-02 CH

PERIOD COVERED

October 1, 1983, to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Nuclear Magnetic Resonance Spectroscopy on Biologically Important Molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Development and application of pulse polarization transfer technique in Nuclear Magnetic Resonance Spectroscopy. Applications include enzyme catalyzed rate studies in vitro and in vivo as well as structural conformational properties of small molecules in solution.

1. Enzyme Catalyzed Exchange

The study of *in vitro* and *in vivo* rate of exchange by NMR spectroscopy is an ongoing project. The *in vivo* unidirectional flux between creatine phosphate and ATP was determined in the leg and head of an anesthetized rat. Using two-dimensional NMR, it was found that the unidirectional flux of creatine phosphate to ATP was 13 μmol/sec/g-weight in the leg and 2 μmol/sec/g-weight in the head. This study represents the first use of two dimensional NMR to study *in vivo* rates of reaction. If one assumes that several cytoplasmic kinases are operating near equilibrium, then it is found that only 10% of the total ADP in the leg or brain is actually free in the cytosol. Using this calculation together with the known substrate, concentrations and the rate constants for the creatine kinase reaction are calculated rates of 8.7 μmol/sec/g-weight in leg and 1.4 μmol/sec g-weight in brain which are in good agreement with the rates observed using two-dimensional NMR. These results indicate that the cytosolic free ADP is much lower than the total ADP determined by extraction procedures, providing that other regulatory mechanisms on the creatine phosphokinase flux are not influencing the reaction.

Comparison of the data available by two dimensional and saturation transfer NMR are currently being made in collaboration with Dr. Balaban. With saturation transfer, it has been shown that very small substrate pools can lead to significant magnetization transfer. The carbonic anhydrase reaction provides an example of this phenomenon where the saturation of an almost invisible carbon dioxide results in the total saturation of bicarbonate. Enzyme complexes can also be saturated as in the case of ADP binding to creatine kinase where saturation transfer experiments on the complex yield the rate of binding ADP to the enzyme. It has been found that using the initial slope method of analyzing the two dimensional rate, that saturation transfer is approximately twice as fast in obtaining comparable signal to noise ratios when a single exchange pathway is being studied. The two dimensional method becomes more efficient as the number of pathways increases. Also, the influence of small pools on phosphate exchange in intact tissues can be determined from a comparison of the two dimensional and saturation transfer experiments. Furthermore, on *in vivo* skeletal muscle of the rat, is was shown that both techniques provide identical results for the exchange between creatine phosphate and ATP catalyzed by creatine kinase.

2. Precision of Nuclear Magnetic Resonance Measurements.

In continued collaboration with Dr. George Weiss (DCRT), a program of estimating the precision of NMR measurements is being developed. In our most recent investigation we determine optimum pulse spacings for measurements of a spin-lattice relaxation times, T_1, when the radio-frequency pulses deviate from their ideal values of 90° and 180°. We also compare the performance of the fast inversion recovery technique in the presence and absence of separate estimates of the values of the rf pulses, using as the criterion the total experimental time required to achieve a specified

precision in the estimate of T_1. We find that where the effective pulse angles are unknown, it is always more efficient to measure them separately on a sample with strong resonances. We also find that the fast inversion recovery method is the most efficient method for estimating T_1 values.

We have also completed an experimental determination of the errors in Nuclear Overhauser Effect measurements on the JEOL FX-60 and Nicolet Magnetics Corporation NT-360 NMR spectrometers. Analysis of these data has included both random and systematic sources of error. We find that it is quite difficult to estimate these factors to better than 5-10% with any degree of certainty.

3. Structural and Conformation Studies using Modern Pulse NMR Methods

The study of actinomycin related peptide lactones in collaboration with Dr. Anthony B. Mauger (Washington Hospital Center) has been completed. The most recent results are concerned with the concentration dependence of the conformer populations in mixed solvents. The techniques used to carry out these studies were homonuclear two dimensional correlated spectroscopy and line shape analysis of the conformer exchange. One of the conformers was found to self associate in non-polar solvents. Molecular models suggest conformation which are held together by two hydrogen bonds as in actinomycin itself. The relationship between this phenomenon and the biological activity is being studied.

In collaboration with Drs. Ad Sax, Nashaat T. Nashed, and Donald Jerina (NIAADK), the assignments of the NMR proton and carbon-13 spectral parameters of a set of fused aromatic hydrocarbons has been completed. The crucial aspect of this study has been the implementation of a new pulse technique which is capable of transferring polarized magnetization through long range coupling constants. For example, in the case of protons coupled to carbon-13, the magnetization from a preselected proton is effectively transferred to carbon-13 nuclei separated from two or three bonds from this proton. A useful application is in determining three bond connectivities in organic molecules since this allows "bridging" of non-protonated carbons as well as nuclei other than carbon such as oxygen or nitrogen. This technique coupled with homonuclear and heteronuclear two-dimensional correlated spectroscopy enabled the complete proton and carbon-13 spectral assignments to be easily obtained. The assignments were carried out on such molecules as benzo (a) anthracene and various fluorinated derivatives, crysene, pyrene, benzo (a) pyrene, benzo (a) napthacene and some related derivatives. This combination of techniques is easy to implement and yields unambiguous results.

A recent study on the mechanism of anomerization of glucose and galactose was completed. It was found that only glucose-6-phosphate and galactose-6-phosphate anomerize at an appreciable ($< 0.1\ sec^{-1}$) rate. A mechanism which implicates the phosphate moiety has been proposed.

Publications

1. Balaban, R. S., Kantor, H. L., and Ferretti, J. A. In vivo flux between phosphocreatine and adenosine triphosphate determined by two-dimensional phosphorus NMR. J. Biol. Chem., 258, 1289 1983.

2. Weiss, G. H. and Ferretti, J. A. Accuracy and precision in the estimation of peak areas and NOE factors. J. Mag. Resonance 55: 397, 1983.

3. Kantor, H. L., Ferretti, J. A., and Balaban, R. S. Kinetics of creatine phosphokinase and adanylate kinase, a two dimensional NMR analysis. Biochim. Biophys. Acta. (in press).

4. Ferretti, J. A., Highet, R. J., Pohl, L. R., Monks, T. J., and Hinson, J. A. Two-dimensional J-resolved nuclear magnetic resonance spectral study of two bromobenzene glutathione conjugates. Environmental Health Perspectives (in press).

Annual Report of the Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for the major red cell disorders. Red cell disorders that produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. In our laboratory investigations, we attempt to focus the most advanced techniques and conceptual knowledge on elucidating the pathogenesis and attempting to devise treatments for these disorders.

Patients with either severe β-thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. During normal human development, HbF ($\alpha_2 \gamma_2$) produced *in utero*, is replaced during the perinatal period with HbA ($\alpha_2 \beta_2$). If both β globin genes are defective either because of mutations that reduce the amount of functional mRNA produced, or because of mutations that result in a structurally abnormal globin (e.g. the β^S globin), the perinatal switch in hemoglobin production leads to the onset of the disease. Reactivation of a γ globin gene in adults could ameliorate the severity of these conditions.

The potential for pharmacological manipulation of HbF synthesis was established when it was shown that 5-azacytidine increases γ globin production in patients with thalassemia and sickle cell anemia. This drug has at least two effects on the rapidly proliferative erythroid precursor population present in patients with thalassemia or sickle cell anemia. First, incorporation of 5-azacytidine into DNA causes a marked reduction in DNA methylation; hypomethylation is characteristic of many expressed DNA sequences. For example, the γ globin gene is undermethylated in fetal liver erythroid cells compared to its complete methylation in adult bone marrow erythroid cells. Second, 5-azacytidine is cytotoxic to erythroid progenitors and, in high doses, can reduce progenitor numbers. Studies described in our individual project "Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe β -Thalassemia and Sickle Cell Anemia" have addressed the mechanism of action of 5-azacytidine. We found that the drug, in low doses, increases assayable progenitor numbers in bone marrow and these progenitors form colonies *in vitro* that contain more HbF than control colonies. Second, exposure of mouse erythroleukemia cells into which human chromosome 11 has been introduced by somatic cell hybridization, to 5-azacytidine results in hypomethylation of human DNA and activation of γ globin gene expression. These two sets of observations suggest that the hypomethylating effect of 5-azacytidine is relevant to its ability to increase HbF synthesis.

That other mechanisms may also be operative derives from the work of others who demonstrated increased HbF synthesis following hydroxyurea or cytosine arabinoside administration. Because hydroxyurea is a safer drug, our clinical efforts have now focused on its evaluation in patients with sickle cell anemia and thalassemia (individual project: "Pharmacological Manipulation of HbF Synthesis). We have learned that

careful monitoring of blood levels and adjustment of drug dose are critical both to avoid toxicity to the bone marrow and to permit induction of HbF synthesis. The most effective regimen used to date is the administration of a single dose weekly (50mg/kg). This schedule has been found effective in increasing release of reticulocytes containing HbF from the bone marrow and following several weeks of administration, to a significant increase in HbF in blood. The drug is also being tested in patients with thalassemia to determine whether the anemia can at least be partially ameliorated, thereby reducing or eliminating a transfusion requirement.

The long term goal of our work is to understand developmental switching in hemoglobin phenotype. We are studying the fine structure of the human γ globin gene promoter to determine whether sequence differences between it and other globin gene promoters are relevant to its selective expression in fetal erythroid cells (individual project: "Regulation of Hemoglobin Switching During Development: Characterization of the Human γ Globin Gene"). Truncation and linker scanning mutants have been created and studied extensively in HeLa cells. An interesting observation is that deletion of one of the two duplicated "CCAAT" sequences leads to increased function of the γ promoter when tested in heterologous cells. This sequence appears only once in β globin gene promoter. The normal β and γ globin genes and the γ globin genes from which one of the two conserved sequences have been removed, have been introduced by DNA transfection into mouse erythroleukemia cells. Other workers have shown that these cells express the human β globin gene but not the human γ globin gene. Thus, we can test whether the removal of one of the two conserved sequences from the γ promoter, thereby making it more resemble the β promoter, leads to an alteration in its expression in these adult erythroid cells.

To obtain further insight into DNA sequences important for the developmental modulation of gene expression, we have studied thalassemia mutants characterized by deletion of varying portions of the β globin gene cluster (see individual project: "Molecular Defects in β Thalassemia"). Several new mutations have been identified. Of interest, is that deletions of quite different size and position appear to produce an identical phenotype on interaction with the β^S gene in doubly heterozygous individuals.

Cellular differentiation and developmental modulation of gene expression reflects the action of intranuclear proteins that interact with specific regulatory sequences around genes. We have devised a strategy designed to identify the action of such proteins on specific DNA sequences and to isolate the genes for these proteins (see individual project: "Enhancer and Promoter Specificity of Immunoglobulin Genes"). Because the promoter and enhancer regions of the immunoglobulin genes have been defined and because these sequences function selectively in myeloma cells, we have chosen to use the immunoglobulin system for our initial studies. The immunoglobulin enhancer and promoters have been linked to the coding sequences of a gene that confers resistance to neomycin. Initial evidence indicating that transacting factors are critical for operation of these hybrid genes has been obtained. L-cells cannot be transformed to neomycin

resistance, but hybrid cells formed between L-cells and myeloma cells, express the immunoglobulin promoter enhancer neomycin resistance recombinant gene effectively. The L-cells may serve as recipients in DNA transfection experiments in which selection to neomycin resistance can be used to identify cells that have incorporated genes for proteins that activate either the enhancer or promoter. Analogous strategies are being devised to identify the protein (and their genes) that modulate expression of the globin genes in specific cells.

Achievement of the differentiated state characteristic of hematopoietic precursors involves the selective and coordinated expression of many genes. Identification of these genes and their gene products is a major objective. Recently, the cellular analogues of viral oncogenes have been proposed as having important roles in cellular differentiation. Hence we have designed studied (see individual project: "Function of Proto-oncogenes in Human Hematopoietic Cells") to examine expression of these genes in bone marrow cells. One gene (fms) is of particular interest in that it is located on chromosome 5 very close to the deletion breakpoint of the $5q^-$ chromosome. The fms gene is known to encode for a transmembrane protein with tyrosine kinase activity; most likely this protein is a growth factor receptor. This deletion chromosome has been found in bone marrow cells of patients with a characteristic syndrome that includes erythroid hypoplasia in abnormal megakaryocytic differentiation. Using specific probes from the fms proto-oncogene in a sensitive S_1 nuclease assay, we have shown that the fms gene is expressed in human bone marrow. Characterization of the fms gene on the $5q^-$ chromosome required the formation of somatic cell hybrids to separate the normal and abnormal chromosome 5 from the bone marrow of these patients. From these studies we have learned that the normal fms gene is deleted from the $5q^-$ chromosome. We speculate that cells having the $5q^-$ chromosome, being hemizygous for the fms locus, having decreased number of receptors for a putative growth factor and therefore these cells proliferate less effectively than normal.

In our effort to understand the regulation of gene expression, we have also chosen to study a gene that is constitutively expressed in all cells in contrast to those genes, such as the globins, that encode for specialized products produced only in red cells. The structure of the human dihydrofolate reductase gene has been determined (see individual project: "Characterization of the Gene for Dihydrofolate Reductase"). This gene has been assigned to chromosome 5 using specific molecular probes and somatic cell hybrids containing varying numbers of human chromosomes. The chromosome assignments of two of the intronless pseudogenes that are part of the dihydrofolate reductase gene family have also been assigned to specific chromosomes. One of the intronless pseudogene is identical in sequence to the coding portions of the normal DHFR genes suggesting a recent evolutionary origin. Study of this gene in humans has revealed a novel form of DNA polymorphism. Some humans have this perfect intronless pseudogene and others do not. Apparently the gene originated following the development of the human species. From the study of the chromatin structure of the DHFR gene has emerged a potential new pattern of gene regulation. The promoter is nucleosome free and hypomethylated whereas the remainder of the gene has the same

structural characteristics as transcriptionally inactive chromatin. Transcription of the DHFR gene is linked to DNA replication. We speculate that the promoter region remains associated with RNA polymerase and other transcriptional proteins but that transcription occurs only when the chromatin structure of the body of the gene is unwound during DNA replication. Thus, there is a link between gene transcription and cell division.

Efficient and reproducible transfer of genes into hematopoietic cells may ultimately prove to be useful for functionally correcting the genetic defects in patients with severe hemoglobinopathies and thalassemia. We have explored the use of DNA viruses to achieve gene transfer into normal hematopoietic cells (see individual project: "Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function"). Recombinant SV40 viruses containing a marker gene, that for chloramphenicol acetyl transferase, has been used to demonstrate efficiency of transfer of genetic information into hematopoietic cells. Stable gene transfer was difficult to achieve because the recombinant SV40 stocks containing a mutant DHFR gene that confers methotrexate resistance, were also contaminated with wild-type SV40. Hence, in collaborative studies, we have used a pure recombinant adenovirus containing a neomycin resistance gene, to demonstrate the feasibility of transfer and stable integration of genes into hematopoietic cells. The ability of such recombinant adenoviruses to transfer genes into bone marrow cells is under active investigation.

Aplastic anemia reflects the total and catastrophic failure of hematopoiesis. The etiology of aplastic anemia remains obscure. Among the potential causes for this syndrome are viral infection, perturbation of the normal cellular interactions essential for effective hematopoiesis, "autoimmune" destruction of hematopoietic stem cells, or an intrinsic abnormality in the stem cells' ability to self renew. The ability of anti-thymocyte globulin (ATG) to partially or completely restore hematopoiesis in 40-50% of patients with aplastic anemia suggest that an irreversible quantitative or qualitative defect in stem cells is not the most common pathogenic mechanism for this disorder.

Our studies have focused on the role of interferon as a potential mediator of hematopoietic suppression in aplastic anemia (see individual project: "Lymphokines in Aplastic Anemia"). Small amounts of α-interferon are present in bone marrow of normal individuals; this interferon is produced by the helper lymphocyte subset. PHA stimulation of lymphocytes results in the release of γ interferon that is highly inhibitory to hematopoietic cell growth. Patients with aplastic anemia often have concentrations of γ interferon that are 10 fold or more than that found in normal bone marrow. A population of suppressor T-lymphocytes, whose activated status is reflected by the presence of HLA-DR and Tac (interleukin-2 receptor) antigens on their cell surface, is found in the peripheral blood of patients with aplastic anemia. In one patient studied to date, production of excess γ interferon and hematopoietic suppressor activity in the bone marrow has been associated with this population of cells. A general hypothesis emerges that γ interferon production by an activated suppressor population of T-lymphocytes may be involved in the pathogenesis of aplastic anemia.

The agent that leads to activation of this suppressor population is of great interest. A parvovirus is known to be involved in one form of bone marrow failure, transient erythroblastopenia in patients with hemolytic anemia. Our study of this model has shown conclusively that a specific virus may cause anemia by infection and destruction of erythroid progenitors. These results have encouraged us to initiate a search for direct evidence of a viral infection in bone marrow cells of patients with aplastic anemia (see individual project: "Viruses and Bone Marrow Failure"). Activated suppressor cells are characteristically present in patients with viral infection whereas increased Tac expression has been observed only in transformed lymphocytes and those infected with the human T-cell leukemia virus.

A recently produced batch of antilymphocyte globulin (ALG), produced in Sweden, has been shown to be inactive in the treatment of patients with aplastic anemia. This unfortunate occurrence has nonetheless provided us an opportunity to compare the immunological properties of active and inactive ALG. The ability of these preparations to stimulate lymphocyte division and hematopoietin production were equivalent. In addition, both the inactive and active forms of ALG contain predominantly IgG that binds to many cell types including lymphocytes, granulocytes, and platelets. The only significant difference uncovered is that the inactive ALG is significantly less able to cause complement-mediated lysis of human T-cells. These results are consistent with a mechanism of action whereby ALG (or ATG) is effective in aplastic anemia by virtue of destruction of an activated suppressor population of T-cells.

In an effort to improve the treatment of patients, the Clinical Hematology Branch is directing a large, multicenter trial of anti-thymocyte globulin in aplastic anemia and related bone marrow disorders (see individual project: "Hematopoiesis in Bone Marrow Failure"). More than 150 patients have been enrolled. Patients with acute severe disease were randomized to receive either ATG for 10 or 28 days; the trend in the data is towards faster and/or more complete responses in patients who received the longer course. ATG has been found to be superior to high dose androgen in patients with chronic severe or moderate aplastic anemia.

Treatment of patients with ATG provides the unique opportunity to study the serum sickness reaction. Many, but not all, patients develop antibodies directed against horse immunoglobulin. The resulting immune complexes provoke a serum sickness reaction with characteristic abnormalities in skin, retina, GI tract and joints. We tested the hypothesis that serum sickness is a necessary requisite for recovery from aplastic anemia following ATG treatment. This hypothesis was disproven. Indeed, 5 of 5 patients who had no clinical or serological evidence of serum sickness recovered from aplastic anemia whereas the recovery rate in those patients who have serum sickness is approximately 40%. These data are consistent with the suggestion that ATG is indeed acting in an immunosuppressive way in inducing recovery of bone marrow function since the lack of formation of anti horse immunoglobulin antibodies will result in a persistently higher titer of horse antibodies cytotoxic to T-lymphocytes following ATG administration.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02203 12 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Defects in Beta Thalassemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

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2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies are designed to define and precisely characterize various molecular lesions occuring in the beta-globin gene cluster in patients with beta thalassemia or with syndromes associated with increased HbF production in adult life such as delta-beta thalassemia, or hereditary persistence of fetal hemoglobin (HPFH). Three new mutations have been characterized. A 12.0 kb deletion removes the δ and β globin genes but spares the moderately repetitive Alu sequences. The phenotype of this mutation on interaction with a β^S gene was found to be identical to the clinical phenotype produced interaction of the β^S with the Ghana type of HPFH-2 deletion. This deletion removes 70 kb of DNA including the Alu moderately repetitive DNA sequences. Thus, very different molecular defects may produce an identical clinical phenotype upon interaction with the β^S globin gene. Two additional mutations, one in a β gene cluster and one in the α gene cluster are being characterized with respect to molecular genotype and clinical phenotype. A previously described mutation that involves two deletions as well as inversion of a 15 kb segment of the β gene cluster has been functionally characterized. The intact $^G\gamma$ gene exhibits features of gene activity, namely undermethylation and DNAseI hypersensitivity but a rearranged hybrid $^A\gamma$ inverted δ gene appears transcriptionally inactive and is partially methylated. To investigate the mechanism by which premature termination codons cause a quantitative deficiency of β mRNA, precursor mRNA molecules are generated in vitro. The processing and nuclear to cytoplasmic transport of these RNA molecules are studied by microinjection into nuclei of Xenopus oocytes.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives: The β-thalassemia syndromes provide an excellent natural model to study the mechanisms of defective gene expression, resulting from point mutations (within or flanking the β-globin gene) or from variable-size deletions that remove important sequences from the β-globin gene cluster. Deletion mutations give rise to particular clinical syndromes that are characterized by an increased production of fetal hemoglobin (HbF) in adult life (δβ-thalassemia and hereditary persistence of fetal hemoglobin, HPFH). The δβ thalassemia and HPFH mutations are clinically distinguished by the phenotype produced in heterozygous individuals. Heterozygotes for δβ thalassemia mutations have 8-15% HbF distributed in 30-50% of their red cells. In contrast, individuals heterozygous for HPFH mutations have 20-30% HbF that is uniformly distributed in all red cells. Individuals with these disorders exhibit a more mild clinical course than those with typical β-thalassemia, due to the beneficial effect of HbF on red cell production and survival. Characterization of the various deletions that have different effects on expression of the γ globin genes may provide insight into the mechanisms that regulate the normal perinatal switch from fetal to adult hemoglobin synthesis. A thorough understanding of these mechanisms may permit novel approaches to manipulation of fetal globin gene expression for treatment of β thalassemia and sickle cell anemia.

Many β^0 thalassemia mutations that cause premature termination of translation also lead to a quantitative reduction in β globin mRNA. Our earlier studies have shown that this quantitative reduction arises because of some alteration in intranuclear processing or nuclear to cytoplasmic transport of the defective mRNA species. Ongoing studies are attempting to elucidate the relationship between the translatability of an mRNA and its intranuclear metabolism.

Methods:

1. High molecular weight genomic DNA from individuals with increased HbF production is extracted from the peripheral white blood cells and is subjected to digestion by restriction endonucleases. The resulting DNA fragments are electrophoresed on agarose gels, transferred to nitrocellulose filters by using the Southern blotting technique and subsequently hybridized with [^{32}P] radiolabeled specific probes (derived by molecular cloning from various regions of the β-globin cluster), and finally autoradiographed. This kind of DNA analysis (called gene mapping) precisely defines the areas and the extent of the deletion mutations in the cluster. DNA was also analyzed in order to determine the frequency of methylated cytosine residues in certain important regions of the globin genes using restriction endonucleases that are sensitive to DNA methylation.

2. Bone marrow cells were extracted using the guanidine hydrochloride technique to recover RNA. The RNAs were analyzed by S_1 nuclease mapping, Northern blotting or spot blotting, using either

nick-translated or uniformly labeled probes, developed in this laboratory.

3. In vitro cultures of peripheral blood erythroid progenitor cells (burst forming units-erythroid, BFU-E) are performed in methylcellulose. The cells are grown either in the presence of standard fetal calf serum or fetal sheep serum, containing a yet uncharacterized "switching factor" that induces switching from fetal to adult hemoglobin synthesis. These studies are performed in collaboration with Drs. Thalia Papayannopoulou and George Stamatoyannopoulos of the University of Washington, Seattle.

4. A vector in which the SP6 bacterophage promoter had been linked to the β globin gene was obtained from Dr. Michael Green of Harvard University. Recombinant DNA methods are used to construct hybrid genes so that specific thalassemic mutations are introduced. SP6 polymerase, commercially available, is used to generate a precursor RNA molecule.

Major Findings:

1. A novel deletion that increases HbF synthesis was found in a Black woman who is doubly heterozygous for this deletion mutation and the β^S (sickle cell) gene. She has mild anemia (Hb 11.6 g/dl, MCV 82 and HbF 27%). Gene mapping defined the 5' endpoint to 2.4 kb (± 0.1 kb) upstream from the δ-globin gene. The two Alu sequences (members of a moderately repetitive DNA sequence family) upstream from the γ globin gene are preserved. The 3' endpoint of the deletion is less than 0.1 kb (±0.1 kb) beyond the end of the β-globin gene. The patient's husband and daughter have a similar clinical syndrome with Hb of 14.5 and 14.1 g/dl and HbF levels of 24% and 25%, respectively. Restriction endonuclease mapping of their DNA demonstrated that each is doubly heterozygous for the β^S gene and the Ghanian type of HPFH-2 deletion; the 5' endpoint of this deletion is in the ψβ gene and it is more than 70 kb long. Both parents had about 99% F-cells (HbF-containing red cells) detected by immunofluoroescence. Additionally, culture of their erythroid progenitors (BFU-E) in the presence of fetal sheep serum rich in "switching factor" resulted in complete suppression of HbF synthesis. This novel δβ deletion resembles the Sicilian type of δβ-thalassemia mutation by its size and preservation of the Alu sequences. However, the clinical and biological phenotype produced by its interaction with the β^S gene is very similar to that of the HPFH type deletion. These data are the first documentation of the phenotypes of compound heterozygote (δβ-thal/ β^S and HPFH/ β^S) where their molecular and cellular phenotype has been fully characterized.

2. A previously reported deletion that produces a $^A\gamma\delta\beta$ thalassemia phenotype has been studied in an Iranian boy who is homozygous for this deletion; he has 100% HbF which is exclusively of $^G\gamma$ type. Gene mapping revealed two areas of deletion, one starting in the second intervening sequence (IVS II) of the $^A\gamma$ gene is about 0.9 kb long and

ends within the exon 3 of the $^{A}\gamma$ gene. Another starts in exon 3 of the γ gene and ends within the 3' end of untranslated area of the β-globin gene. Additionally, the remaining DNA sequence between the $^{A}\gamma$ and β genes is inverted, so that exon 3 of the δ gene is "fused" with the truncated IVS2 of the $^{A}\gamma$ gene and the end of exon 3 of the $^{A}\gamma$ gene is translocated in the vicinity of the 3' untranslated region of the totally deleted β-globin gene. The ε and $\psi\beta$ genes were totally methylated while the 5' end of the γ gene and the region downstream from the missing β globin gene were partially methylated. On the contrary, the $^{G}\gamma$ genes and the intact 5' end of the $^{A}\gamma$ gene were not methylated. In addition to undermethylation, another feature of gene expression is the presence of DNAseI hypersensitive sites at the 5' end as demonstrated by exposure of intact nuclei to nuclease. Both the promoters of the normal $^{G}\gamma$ and the $^{A}\gamma$ fused δ globin genes are DNAseI hypersensitive. The δ globin gene promoter, hypersensitive in adult bone marrow cells, lack sensitivity in the chromosome with the inversion deletion mutation.

S_1 nuclease mapping showed normal splicing and processing of $^{G}\gamma$ mRNA. Current studies involve search for transcriptional activity of the abnormal fusion gene in either direction, by employing Northern blot analysis, RNA spot blot analysis, and S_1 nuclease mapping of RNA using uniformally labelled δ probes of sense (5' to 3') and nonsense (3' to 5') orientation.

The biosynthetic pattern of erythroid colonies derived from BFU-E of the patient showed a normal response to "switching factor in fetal sheep serum (FSS). When cultured in fetal calf serum, the α/γ globin biosynthetic ratio was 2.0 but when BFU-E were grown in FSS, the ratio increased to 4.0-5.0 indicating that the erythroid progenitors became more markedly thalassemic as expression of the γ gene was suppressed. Despite the major disruption in the globin gene cluster, the erythroid progenitors responded to an external stimulus by assuming a more adult, in this case thalassemic, phenotype.

3. Characterization of a new deletion producing the $\delta\beta$ thalassemia phenotype is in progress. This was found in a rather healthy mother of German extraction who exhibited 9% HbF and 90% F-cells with a normal $^{G}\gamma$ / $^{A}\gamma$ ratio of 2:3. The molecular data so far are consistent with a deletion starting about 1.5 kb upstream from the $^{A}\gamma$ gene and extending at least 68.0 kb downstream. Study of this mutation suggests an intriguing possibility, namely that there may be a trans-acting effect of this deletion on the normal chromosome since deletion of one $^{A}\gamma$ gene is nontheless associated with a normal ratio of $^{A}\gamma$ gene and $^{G}\gamma$ synthesis.

4. A new deletion in the α globin gene cluster has been discovered in a Black individual with HbH disease and sickle cell trait. The deletion is at least 27 kb long and removes both alpha genes but

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leaves the zeta genes intact producing the very rare (--/) chromosome of alpha thalassemia-1 in Blacks. This is the second deletion reported as the molecular basis for the rare alpha thalassemia-1 phenotype.

5. Characterization of the intranuclear metabolism of the globin mRNA precursor transcribed from a β^0-39 thalassemia are in progress. A recombinant in which the portion of the β globin gene containing the β^0-39 codon has been substituted for the corresponding portion of the normal β globin gene has been constructed. Hence, we now have a gene with the SP6 promoter fused to the coding portions of the β^0 thalassemia gene with a premature termination codon in position 39. The transcriptional assays have been optimized so as to generate large amounts of both the normal and β^0-39 transcript for injection into Xenopus oocytes.

Significance to Biomedical Research and the Institute Program:

Homozygous β-thalassemia and the related disorders of increased HbF synthesis in the adult life are among the more common genetic diseases and may cause morbidity to affected individuals who are homozygous for these mutations. Molecular analysis and thorough characterization of these deletions and point mutations allows understanding of the genetic basis of these diseases and also will provide new insights into molecular mechanisms involved in the expression of genes in human cells.

Proposed Course of the Project:

Molecular characterization of the new deletion mutations in either the β or α gene cluster will be completed. Our efforts during the next several months will focus on characterization of the β^0-39 mutation. In addition to the studies described above, the normal and β^0 thalassemia genes are being introduced by DNA transfection, into mouse erythroleukemia cells. The resulting transformed cell lines will provide an opportunity to more thoroughly characterize the intranuclear metabolism of the defective β^0-39 gene transcript and to determine whether its quantitative deficiency may be corrected by introduction of a specific suppressor transfer RNA gene. The gene to be used encodes for a tRNA that suppresses the stop codon at the β^0-39 position by inserting an amino acid into the globin polypeptide chain.

Publications:

1. Nienhuis, A.W., Anagnou, N.P., Ley, T.J.: Advances in thalassemia research. Blood 63:738-758, 1984.

2. Humphries, R.K., Ley, T.J., Anagnou, N.P., Baur, A.W., and Nienhuis, A.W.: β^0-39 thalassemia gene: a premature termination codon causes β mRNA deficiency without affecting cytoplasmic β mRNA stability. Blood 64:23-32, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02208 10 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Iron Chelation in Transfusional Hemochromatosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
Others: Patricia Griffith, Clinical Nurse Specialist, CHB, NHLBI
Timothy J. Ley, M.D., Senior Investigator, CHB, NHLBI
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H. Strawczynski, M.D., Director, Chronic Care Clinic, Montreal Children's Hospital, Montreal, Quebec, Canada

COOPERATING UNITS (if any) Laboratory of Molecular Biology, NHLBI, NIH, Bethesda, Maryland
Cleveland General Hospital, Division of Hematology, Cleveland, Ohio
Cardiology Branch, NHLBI, NIH, Bethesda, Maryland
Montreal Children's Hospital, Montreal, Quebec, Canada

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferrioxamine is administered by subcutaneous infusion and iron removal is determined by quantitation of urinary iron excretion and careful recording of the total iron administration by transfusion. Over the past four years, 49 patients have participated in a randomized study to evaluate the safety of low dose ascorbic acid. Thirty patients took the desferal and ascorbic acid regularly for four years and therefore were available for evaluation. Randomization was successful in that the two groups were equivalent. No evidence of ascorbic acid toxicity was noted and therefore we concluded that low doses of the vitamin may be safely given to enhance urinary iron excretion in young patients with thalassemia. During the past year we have initiated a collaboration with Dr. Gary Brittenham of the Cleveland Clinic. Using magnetic susceptability, Dr. Brittenham can estimate liver iron concentration accurately. This quantitative measurement will be obtained in our entire group of 65 patients and these data correlated with the amount of desferal administered and other clinical measurements of iron overload.

Project Description:

Objectives:

The objectives of these studies are to evaluate iron chelators, to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to develop clinical criteria that will be helpful to determine the efficacy of chronic chelation therapy. A randomized trial of supplemental ascorbic acid was initiated to assess the value and/or toxicity of this agent in promoting mobilization of iron by desferrioxamine in patients with iron overload.

Methods:

Patient populations that participate in these studies include: 1) patients with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life, and 2) patients with idiopathic hemochromatosis at various stages in the process of iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: An estimate of cardiac size by chest x-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function is assessed by resting ejection fraction. In selected patients, radionuclide angiography is performed at rest and at exercise to determine the functional reserve of the left ventricle.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. The liver iron concentration is determined non-invasively based on magnetic susceptability measurements (N Eng J Med 307:1671-1675, 1983).

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

Major Findings:

The results of the randomized trial of ascorbic acid have been analyzed. Of the 49 patients who initially were enrolled in this study, only 30 used the subcutaneous desferrioxamine and took the ascorbic acid at least five days per week for four years. Sixteen patients had been randomized to ascorbic acid and fourteen to placebo. The two groups were clinically equivalent. No evidence of cardiac or other toxicity was evident on detailed clinical testing. We conclude therefore that ascorbic acid, in low dose may be safely given to patients on regular chelation therapy.

Evaluation of our total group of 65 patients with congenital anemia on the chelation protocol revealed a broad range of compliance with the desferrioxamine regimen. Over the next several months, each patient will have a quantitative measure of liver iron concentration. This parameter will be correlated with desferrioxamine use as determined by the total number of vials of desferal provided to the patient over the last five years. Correlation between compliance and liver iron concentration will be assessed. The incidence of other abnormalities, particularly non-invasive and clinical cardiac assessment, will be evaluated.

Significance to Biomedical Research and to Institute Program:

Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project:

This project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion therapy in thalassemia and other congenital hemolytic anemias is removed. Within the next three or four months we should be able to judge, based on our clinical experience, whether there is evidence of efficacy of the current chelation regimen. Selected patients with clinical evidence of cardiac hemochromatosis will receive intensive intravenous therapy in an effort to reverse cardiac disease.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02304 07 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hematopoiesis in Bone Marrow Failure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others: Pedro Gascon, M.D., Medical Staff Fellow, CHB, NHLBI
Jeffrey Moore, B.A., Chemist, CHB, NHLBI
R. Keith Humphries, M.D., Visiting Associate, CHB, NHBLI
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Clinical Hematology Branch

SECTION

Section on Cell Biology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Insights into the regulation of normal and depressed hematopoiesis have been provided by the study of patients with bone marrow failure and their tissues. Laboratory studies have focused on three aspects of bone marrow depression: 1) the cell biology of hematopoietic progenitors; 2) immunological aspects of normal and aberrant bone marrow regulation, especially the role of lymphokines in stimulation and suppression of bone marrow cell growth (see Individual Project: Lymphokines in Aplastic Anemia, # Z01 HL 02315 02 CHB); 3) the role of viruses as etiologic agents in aplastic anemia (see Individual Project: Viruses and Bone Marrow Failure, #Z01 HL 02319 01 CHB). In an effort to improve the treatment of patients, the Clinical Hematology Branch is directing a large, multi-center trial of anti-thymocyte globulin in aplastic anemia and related bone marrow disorders. More recently we have investigated the use of Acyclovir, an anti-viral agent, in patients with aplastic anemia.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives and Methods:

The peripheral blood granulocytes, platelets, and red blood cells are continuously produced throughout life by bone marrow stem cells that interact with regulatory lymphocytes and humoral factors. Bone marrow is readily obtained under local anesthesia with minimal risk to the patient, and furthermore bone marrow progenitor cells can be quantitated by growth in vitro in semi-solid media. Individual progenitors will form colonies with characteristic morphology under the appropriate conditions of temperature and humidity with the addition of specific growth factors and non-specific nutrients; these include fetal calf serum, albumin, erythropoietin, burst promoting activity, or colony stimulating factor. The last two are derived from leukocyte conditioned media.

Erythroid and myeloid hematopoietic progenitors are severely reduced in patients with aplastic anemia, corresponding to the reduction in bone marrow production that leads to pancytopenia. Pancytopenia is a major feature of acute leukemia as well, and some parallels can be drawn between leukemia and aplastic anemia. Leukemia is believed to be the result of a "block" in the differentiation of hematopoietic cells at a late stage, resulting in a population of mitotically active cells with limited long-term proliferative capacity. We have theorized that aplastic anemia, which results in similar symptoms but a strikingly different bone marrow morphology, may be the result of a similar block in differentiation. A block at an earlier stage in stem cell maturation, at which cells have an enormous proliferative capacity but are only occasionally in mitotic activity, would result in a paucity of visible cells in the bone marrow. Alternatively, an apparent block in differentiation may be the result of an abnormality in cell proliferation. For example, cells which have a continued stimulus for proliferation may be unable to differentiate (leukemia); alternatively, cells which fail to proliferate may differentiate leading to extinction of the population. In either case, under some conditions stem cells may be rescued, and because of their high proliferative capacity, normal hematopoiesis re-established.

Rescue of the most primitive stem cells is possible in a proportion of patients with aplastic anemia treated with anti-thymocyte globulin. The number of responding patients varies from between 40 and 50%. Most observers believe that anti-thymocyte globulin (ATG), a horse serum preparation directed against human thymocytes, is immunosuppressive, and that bone marrow failure in aplastic anemia may be the result of an "autoimmune" type of inhibition of hematopoiesis. Alternatively, anti-thymocyte globulin may be functioning by a stimulatory, as opposed to inhibitory action, on residual lymphocytes in patients with bone marrow failure. Studies on the mechanism of action of ATG are described in annual report #ZO1 HL 02315 01 CHB.

ATG has been employed in a number of regimens and using a number of preparations. Either thymocytes or lymphocytes (obtained from thoracic duct drainage) have served as immunogens; varying immunization regimens have been employed in different breeds of horses; and treatment regimens in patients have varied widely, not only in dose and timing, but also in concurrent use of androgens, corticosteroids, and haploidentical, mis-matched bone marrow transplantation. Our branch currently heads a large, multi-center trial that plans to enroll 200 patients with aplastic anemia and related bone marrow disorders to determine appropriate dose regimens and the range of diseases responsive to ATG. Patients with acute severe aplastic anemia, as determined by blood count criteria, are randomized to receive ATG in two dose regimens (15 mg/kg/day x 10 days or 15 mg/kg/day x 14 days followed by 15 mg/kg/every other day x 14 days). Patients with moderate aplastic anemia or with severe disease of longer than twelve weeks' duration are randomized to receive ATG for 10 days or high doses of Deca-Durabolin, an androgen. In a third group, patients with a variety of bone marrow failure syndromes including myelofibrosis, pancytopenia with hypercellular bone marrow, pure red cell aplasia, immune panleukopenia, and amegakaryocytic thrombocytopenia, receive ATG for ten days.

Viruses may be important etiologic agents in producing bone marrow failure in some patients. Non-A non-B hepatitis and infectious mononucleosis are occasionally followed by aplastic anemia. In animals, a number of viruses are capable of causing bone marrow hypoproliferation. We have studied a virus recently associated on epidemiologic grounds with transient aplastic crises of chronic hemolytic anemias. Patients with sickle cell disease, hereditary spherocytosis, and other hemolytic processes often suffer during their childhood a brief interruption of erythropoiesis, which results in marked, temporary worsening of their anemia. From clinical observation, aplastic crises have been thought secondary to viral illness. Recently, the discovery of a serum parvovirus and the development of immunological methods for its detection have allowed the demonstration that patients with such transient aplastic crises have either parvovirus antigenemia or develop IgM antibodies to this virus. In vitro, serum containing the virus has been mixed with bone marrow cells and assayed in the tissue culture system described above. The specific nature of virus interaction with bone marrow progenitor cells has been determined, using replating methods which isolate progenitor cells at defined periods of differentiation. Conventional cytochemistry, electronmicroscopy, and immunofluorescence have been used to identify cells infected with parvovirus. Further studies aimed at discovering new viruses or defining the role of viruses in hematopoietic failure have included measurement of immune complexes and interferon levels in patients with aplastic anemia, screening of sera for viral antibody specificities, and treatment of patients with Acyclovir, an anti-viral agent.

Results:

Cell Biology: Patients with aplastic anemia have markedly decreased numbers of committed hematopoietic progenitor cells. In the mouse, truly undifferentiated progenitors form blast colonies which can be cultured in methylcellulose for up to 45 days and are capable of extensive colony formation on replating at the end of 3 weeks.

We have been unable to culture blast colonies with high proliferative capacity from either patients with normal bone marrows or those with aplastic anemia. However, long-term tissue cultures from normal individuals and patients with aplastic anemia have been established in suspension systems in an effort to amplify the number of cells capable of blast colony formation by promoting self-renewal *in vitro*.

ATG Treatment of Aplastic Anemia: The multi-center trial has enrolled over 150 patients as of July 9, 1984. Data have been collected and placed on computer disks for statistical analyses. Preliminary analysis of patients in Group I, with acute severe disease who are randomized to receive either ATG for 10 or 28 days, are summarized below. While there is no statistical difference in overall transfusion independence in patients with severe disease treated with either regimen, the trend is towards faster and/or more complete responses in patients who receive the longer course of antithymocyte globulin. Preliminary data on patients in Group II, with chronic severe or moderate aplastic anemia, strongly suggest that ATG is superior to Deca-Durabolin at a p value of approximately 0.1. ATG has only been sporadically successful in the treatment of patients with a variety of other bone marrow depression syndromes, in particular, paroxysmal noctural hemoglobinuria and myelodysplasia.

Viruses and Hematopoiesis: The serum parvovirus is a potent inhibitor of CFU-E derived colony formation *in vitro*. Replating experiments have established the erythroid progenitor, and not an accessory cell, as a target of the virus. Inhibitory activity co-purifies with virus on sucrose density gradients and is separable from the immunoglobulin and interferon components of serum. There is no evidence for a helper virus effect. Although the virus has the physicochemical properties of a parvovirus, it is somewhat more heat sensitive than most viruses of this class. However, an immunosupressive strain of parvovirus in mice is similarly heat sensitive. Parvovirus infected cells develop nuclear inclusion bodies. The parvovirus has been directly visualized in erythroid progenitor cells by electronmicroscopy, and virus antigen is detectable in erythroid colonies grown *in vitro* by immunofluorescence using a monoclonal antibody.

Immunofluorescent assay for the human parvovirus in infected cells has been developed. Current efforts to promote the growth of the cell

in culture include use of adenovirus infected cell lines. We have investigated a large outbreak of fifth disease and transient aplastic crisis in Cleveland in collaboration with the Communicable Disease Center, and found a perfect correlation between the occurrence of parvovirus antigen in blood and complete inhibition by serum of erythropoietic activity in vitro. The parvovirus studies have been expanded to include laboratory studies of feline panleukopenia virus in cats, commonly thought to be an etiologic agent for true aplastic anemia in this species.

The search for retrovirus in patients with aplastic anemia is described in individual report (Z01 HL 02319 01 CHB).

Proposed Course of the Project:

Efforts to detect human blast colonies in vitro will continue.

A statistical analysis of the multi-center trial will be complete for Group I in six months and for Group II in one year.

With the development of the immunofluorescent assay parvovirus, an attempt will be made to culture the human parvovirus in vitro using a variety of test cell lines. The parvovirus has been molecularly cloned, and with these clones now available, an effort will be made to identify parvovirus DNA sequences in the normal human genome as well as in patients with aplastic anemia.

Four patients have been treated with the antiviral agent Acyclovir. Transfusion independence has been observed following treatment in one patient, and markedly improved granulocyte counts in two others.

Publications:

1. Mortimer, P.P., Humphries, R.K., Moore, J.G., Purcell, R.H., and Young, N.S.: A human parvovirus-like virus inhibits haematopoietic colony formation in vitro. Nature 302:426-429, 1983.

2. Young, N.S., Mortimer, P.P., Moore, J.G., and Humphries, R.K.: Characterization of a virus that causes transient aplastic crisis. J. Clin. Invest. 83:224-230, 1984.

3. Young, N. and Mortimer, P.: Viruses and bone marrow failure. Blood. 63:729-737, 1984.

4. Young, N., Moore, J., and Humphries, R.K.: The human parvovirus and in vitro colony formation. In, "Aplastic Anemia: Stem Cell Biology and New Clinical Concepts." (Eds. Young, N., Levine, A., and Humphries, R.K.). Alan R. Liss, New York, pages 221-226, 1984.

5. Pappas, S.C., Hoofnagle, J.H., Young, N.S., Straus, S.E. and Jones, E.A.: Treatment of chronic non-A, non-B hepatitis with Acyclovir: pilot study. J. Med. Virology. In press.

OUTCOME IN GROUP I

RANDOMIZATION GROUP		OUTCOME				
FREQUENCY PCT		DIED WKS 0-8	DIED WKS 8-	FAILURE TO RESPOND	TRANSFUSION INDEPENDENT	
ATG(10d)	3	6	3	11	12	32
	.	18.75	9.38	34.38	37.50	
ATG(28d)	3	8	2	8	15	33
	.	24.24	6.06	24.24	45.45	
TOTAL	.	14	5	19	27	65

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02307 05 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: Keith Humphries, M.D., Visiting Associate, CHB, NHLBI
Amanda Cline, Research Assistant, CHB, NHLBI
Austine Moulton, Research Assistant, CHB, NHLBI
Arthur Nienhuis, M.D., Branch Chief, CHB, NHLBI
Yakov Gluzman, M.D., Cold Spring Harbor Laboratory, New York
Kevin Van Doren, M.D., Cold Spring Harbor Laboratory, New York

COOPERATING UNITS (if any)

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LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	3.0	1.0

CHECK APPROPRIATE BOX(ES)

X (a) Human subjects X (b) Human tissues ☐ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The immediate goal of these studies is to develop methods for efficiency introducing human globin genes into hemopoietic cells both from tissue culture lines and normal bone marrow to study their tissue specific regulation. We have constructed a hybrid SV40 virus which contains the prokaryotic gene coding for chloramphenicol acetyl transferase (CAT) and have obtained gene transfer and transient expression of CAT in a large number of fibroblast and hemopoietic cell lines of both mouse and human origin and in normal fresh bone marrow cells of mouse, monkey and man. The results with suspension hemopoietic cell lines and fresh bone marrow cells represent a significance advance over the low or undetectable levels of gene transfer obtained with the CaPO4 precipitate technique. We have also constructed an SV40 recombinant containing the methotrexate resistant DHFR coding sequence and have successfully used this viral lysate to transform CHO DG21 cells that lack both DHFR genes. Recombinant SV40 lysates contain wild type SV40 virus that severely compromises their use as viral vectors. We have therefore made use of helper free recombinant adenoviruses that contain the neomycin resistant gene and have successfully transformed both K562, MEL and various fibroblast cell lines to G418 resistance. In K562 cells 1-3 copies of the whole virus are found to be integrated. As the transformation frequency of adenovirus in K562 and MEL cells is higher than that of calcium phosphate, mediated DNA transfer, viral vectors will be constructed to facilitate introduction of a selectable gene and a human globin gene into both hematopoietic cell lines in vitro and normal hematopoietic stem cells in vivo.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives:

The goal of correcting human genetic defects which result in severe disease by genetic therapy is becoming an increasingly more realistic objective based on substantial knowledge about gene structure and function derived from exploitation of the molecular cloning and recombinant DNA technology. Much remains to be learned about the specific regulatory sequences which enable the high level expression of globin genes in a stage and tissue specific manner. Needed are efficient methods for introducing globin genes into hemopoietic cell lines. $CaPO_4$ mediated DNA transfer techniques are very inefficient for cells which grow in suspension and for nontransformed normal cells. We have successfully used an SV40 recombinant containing the chloramphenicol acetyl transferase (CAT) gene to introduce the CAT gene into various hemopoietic and other cell lines as well as bone marrow cells from mouse, monkey and man. An SV40 recombinant containing the DHFR methotrexate resistant gene has successfully been used to transform CHO DG21 cells to methotrexate resistance. Furthermore, adenoviral recombinants that contain the neomycin resistance gene have successfully been used to transform both K562 and MEL cells to G418 resistance. The adenovirus can easily carry two genes separated by 20 kilobases of relatively inactive DNA and is therefore an ideal viral vector to introduce two genes, e.g. a human globin gene and a selectable marker at the same time. Viral vectors containing a human globin gene and a selectable marker are now under construction. They will be used to transform hemopoietic cell lines and also bone marrow from mouse, monkey and man in an attempt to transfer globin genes stably into these cells.

Methods:

1. Construction of hybrid SV40 viral genomes: All constructions are performed by use of the plasmid vector pBR322 or pUC9 to allow cloning of constructs into E.coli. The principle of the constructions is to delete a portion of either the early or late region of SV40 and replace it with the selectable marker gene or globin gene by recombination and molecular cloning. The recombinant viral genome is released from the plasmid vector by restriction endonuclease digestion and recircularized by incubation with DNA ligase.

2. Lytic infection of monkey kidney cells: Hybrid viral DNA is mixed with DNA from a complementary helper virus defective in either the early or late region. The mixture is introduced into permissive monkey kidney cells. Following complete lysis of the cells a viral stock is harvested which is used in turn to generate a high titer secondary viral stock for further use.

3. Transformation of tissue culture or bone marrow cells with DNA or recombinant virus: For transfection by virus, a high titer viral suspension is added directly to cell monolayers or to suspension cells at high concentration. For comparison, purified vector DNA is precipitated by the calcium phosphate technique and incubated with the cells for approximately four hours prior to removal of the precipitate and addition of fresh media. After the initial transfection period, the cells can be incubated in vitro under a variety of culture conditions and in addition in the case of bone marrow cells from mouse and monkey, may be reinjected into lethally irradiated recipients.

4. Analysis of gene expression. To assess transient expression of the CAT gene, cells are harvested at various times after transfection and a cell extract prepared by lysis and precipitation of membrane components. Following incubation with the substrate [^{14}C]-chloramphenicol, acetylated products are resolved using thin layer chromatography. For analysis of globin gene expression, RNA can be harvested for 5' mapping using probes specific for globin mRNA sequences. Oligo nucleotide primers have been obtained to permit detection and analysis of CAT gene and globin gene expression by "primer extension" RNA analysis.

5. Adenoviral recombinants that lack both the E1 gene and the E3 gene including the transforming E1a gene are constructed by placing the insert either in the E1 or E3 region of the virus. This can be accomplished either by in vitro ligation of the insert and the two viral arms followed by transfection into 293 cells, or the construction can be done by recombination then by cotransfecting into 293 cells two viral fragments (one containing the insert) with sufficient overlapping sequences to allow recombination. 293 cells that express the E1a protein can replicate the otherwise replication defective recombinant virus and a helper free pure recombinant virus can thereafter be plaque purified, grown up and analyzed by restriction enzyme analysis.

Results:

1. A recombinant SV40 virus in which the early region had been replaced with a chloramphenicol acetyl transferase [CAT gene driven by the promoter from Rous sarcoma virus LTR], transfers the CAT gene more efficiently into mouse and human bone marrow cells and into various tissue culture cell lines that grow in suspension than the classical calcium phosphate DNA transfer procedure as shown by assay for CAT activity 48 hours after infection.

2. Recombinant SV40 virions stably transform Chinese hamster ovary cells to methotrexate resistance by use of an early region recombinant containing the methotrexate resistant dihydrofolate reductase (DHFR) gene driven by the RSV promoter. The entire DHFR transcriptional unit could be detected in the genome of transformed cells that are also shown to be resistant to methotrexate.

3. Due to a difficulty to obtain helper free SV40 recombinants we have made use of a helper free adenovirus recombinant containing the neomycin resistance gene. This adenovirus recombinant that contains the neomycin resistance gene driven by the SV40 early promoter has been used to transform K562 and MEL hematopoietic cell lines to resistance to the antibiotic G418. Transformation frequency was much higher than that obtained with calcium phosphate precipitated DNA. Most or all of the recombinant adenovirus genome was integrated as 1-3 copies in the transformed cells. These studies show the feasibility of using adenoviruses for introduction of new genetic material into hemopoietic cells.

Significance to Biomedical Research in the Institute Program

Many serious human diseases arise because of monogenetic mutations affecting the structural or enzymatic component within cells. Two of these in which we are particularly interested are sickle cell anemia and homozygous β thalassemia. The goal of achieving genetic therapy for these conditions should be obtainable with increased knowledge of globin gene regulation and the development of gene transfer methodology. In the course of pursuing this objective additional knowledge will be gained regarding the function of transcriptional regulatory signals in eukaryotic cells.

Proposed Course of the Project

1. Adenovirus recombinants containing two genes, a globin gene and a selectable marker are now under construction. These double recombinants contain a human globin gene and one selectable marker, either the neomycin resistance gene to transform tissue culture cells or the DHFR methotrexate resistance gene to use for selection in vivo.

2. Adenovirus double recombinants containing a globin gene and a neomycin resistance gene will be used to transform K562 cells, MEL cells and possibly other hematopoietic tissue culture cells. High molecular DNA from the transformants will thereafter be analyzed by Southern blotting to identify globin gene sequences and gene expression of the transferred globin gene will thereafter be analyzed by S_1 mapping or primer extension.

3. Adenovirus double recombinants containing the methotrexate resistant DHFR gene will be used to infect bone marrow cells from mice and monkeys and autologous bone marrow transplantation will be performed when the animal has been irradiated to kill its remaining stem cells. Transformed bone marrow cells will subsequently be selected by giving methotrexate in vivo.

4. The adenovirus double recombinants containing the neomycin resistance gene will be used to study globin gene regulation. Hybrid globin genes, mutant genes, deletion mutants, and linker scanning mutants can be introduced into an adenovirus vector to study gene regulation and expression of the inserted gene.

5. Explore the use of various retroviruses to transform stem cells in the bone marrow of mouse, monkey and man.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02310 04 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Gene for Human Dihydrofolate Reductase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Takashi Shimada, M.D., Visiting Fellow, CHB, NHLBI

Others: Austine Davis Moulton, Research Assistant, CHB, NHLBI
Maria Harrison, Electron Microscopist, CHB, NHLBI
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

DNA methylation and chromatin structure of the constitutively expressed normal and amplified human dihydrofolate reductase (DHFR) gene were studied. Only the 5' promoter region of the DHFR gene was found to be undermethylated, while the remaining 30 kilobase (kb) gene was completely methylated. The promoter region was DNaseI hypersensitive in chromatin. In methotrexate (MTX) resistant HeLa cells with an amplified DHFR gene, all copies of the amplified gene exhibited a pattern of undermethylation and DNaseI hypersensitivity of only the promoter region. Detailed mapping of the DNaseI hypersensitive sites revealed five discrete cutting sites; two were also sensitive to S_1 nicking when this DNA fragment was part of supercoiled plasmid DNA. DNA mediated gene transfer showed that in vitro methylation of the promoter region markedly reduced transformation frequency of $DHFR^-$ CHO cells. The DHFR gene fragment in cells having the $DHFR^+$ phenotype had become specifically demethylated in the promoter region. Chromosomal organization of the DHFR gene family was studied by DNA analysis of human X rodent somatic cell hybrids. The functional gene and five intronless pseudogenes were found to be dispersed to different chromosomes. The functional gene was assigned to chromosome 5 and two pseudogenes, hDHFR ψ2 and ψ 4 to chromosomes 6 and 3, respectively. A intronless pseudogene with perfect sequence homology to the functional locus (hDHFR-ψ1) was found to be polymorphic in that it is present in DNA from some individuals but not others.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Objectives:

One objective of this study is to compare the mode of regulation of constitutively expressed genes to the mechanism of regulation of the genes which encode for products characteristic of the differentiated state. Constitutively expressed genes are generally crucial for cell proliferation and growth while specialized genes are most relevant to the development of the differentiated state. Dihydrofolate reductase (DHFR) was chosen for study because of its essential role in cell metabolism; it is required for the synthesis of deoxynucleotide precursors utilized for DNA synthesis.

The second objective of these studies is to characterize amplification of the DHFR gene. Growth of cultured cells in methotrexate, a selective inhibitor of dihydrofolate reductase, often leads to amplification of the DHFR gene. Our purpose is to compare the structure and organization of functional DHFR genes in normal and methotrexate resistance cells and thereby to deduce certain features relevant to the mechanism of gene amplification.

We have cloned and characterized the functional human DHFR gene and three intronless DHFR pseudogenes. The functional gene is about 30 kb long and consists of six exons and five introns. A single transcriptional start site and three polyadenylation sites have been identified; these account for the 0.8, 1.0, and 3.8 nucleotide DHFR mRNA species in human cells.

The promoter region of the human DHFR gene is extremely rich in guanosine and cytosine residues. Its sequence is highly homologous to the corresponding region of the mouse DHFR gene. In addition, this promoter was shown to function without an exogenous enhancer element when tested in a transient assay in monkey kidney and HeLa cells.

We have now studied the chromatin structure of the normal and amplified DHFR gene and chromosomal organization of the DHFR gene family.

Methods:

1. Methylation pattern of the DHFR gene: The methylation pattern of specific sites within the DHFR gene was assayed using the methylation sensitive restriction endonuclease HpaII, MspI or HhaI. DNA samples from normal and MTX-resistant cells were digested with these enzymes and the pattern of digested fragments was analyzed by the Southern blot technique.

2. DNaseI hypersensitivity: Isolated nuclei from K562 cells or MTX-resistant HeLa cells were treated with increasing amounts of DNaseI. DNA extracted from these nuclei were restricted with Hind III or EcoRI

and analyzed by Southern blotting using probes for either end of the restricted fragments.

3. S_1 nuclease sensitivity: The 1.8 kb EcoRI fragment containing the 5' end region of the DHFR gene was subcloned into pBR322. The supercoiled plasmid DNA was treated with S_1 nuclease and subsequently restricted with EcoRI. Released discrete bands on an agarose gel were detected by ethidium bromide staining or Southern blot analysis.

4. In vitro DNA methylation and transformation: A DHFR mini gene containing 1.2 kb of 5' flanking sequence, the first intron, and all of the exons was methylated in vitro using HpaII methylase or HhaI methylase in the presence of S-adenosyl methionine. The methylated DNA was introduced into $DHFR^-$ CHO cells by the standard calcium phosphate precipitation technique and $DHFR^+$ transformants were selected in medium lacking glycine, hypoxanthine, and thymidine.

5. Gene mapping: Genomic DNA was prepared from rodent and human somatic cell hybrids that contained several human chromosomes in different combinations. These DNA samples were digested with EcoRI and blot hybridized with the DHFR coding sequence probe or the intron probe which is specific for the functional human DHFR locus.

Major Findings:

1. The human DHFR gene was found to be undermethylated only in its 5' promoter region. The remaining CCGG residues in the 30 kb DHFR gene were resistant to digestion by HpaII. Each of 27 CpG residues that were part of an HpaII or HhaI cutting site within a 1.1 kb segment of the DHFR gene promoter region were found to be undermethylated.

2. The undermethylated promoter region was shown to be DNaseI hypersensitive in chromatin. The remainder of the gene is insensitive to DNaseI digestion and no other hypersensitive sites were present within and for at least 5 kb downstream of the DHFR gene.

3. In MTX-resistant HeLa cells containing roughly 80 copies of the DHFR gene, all copies exhibited a pattern of undermethylation and DNaseI hypersensitivity of only the promoter region. These results suggest that all gene copies are transcriptionally active.

4. Detailed mapping of the DNaseI hypersensitive region revealed four strong cutting sites within a 500 bp segment immediately upstream from the DHFR coding sequence and a weak site within intron I. Three major sites fall within the block of DHFR sequences that are homologous to the corresponding region of the mouse genome, suggesting that this region is important for regulation of DHFR gene expression.

5. Two of the major DNaseI cutting sites in chromatin were also sensitive to S_1 nuclease nicking when this DNA fragment was part of supercoiled plasmid DNA. These results suggest that hypersensitivity of these two sites in chromatin reflect an intrinsic property of the DNA sequence.

6. The DHFR mini gene that was methylated in vitro was only 10-20% as efficient in transforming CHO DHFR$^-$ cells as the unmethylated mini gene. Cells transformed by methylated DNA were characterized by DNA extraction and Southern blotting. Only a very restricted region of the gene immediately upstream from the start site of transcription had become undermethylated on transformation of these cells to the DHFR$^+$ phenotype. The nucleotides upstream, normally undermethylated in cells containing the endogenous gene, remain completely methylated in the CHO transformants. Nonetheless, the entire 500 bp of sequences immediately upstream from the start site of transcription exhibited DNaseI hypersensitivity.

7. One functional and four intronless pseudo DHFR genes were dispersed in that each one was found on a different chromosome. This evidence support the proposed mechanism of formation of these intronless genes, namely conversion of an mRNA molecule into DNA and reinsertion into a chromosome.

8. Detailed gene mapping showed the functional DHFR gene to be on chromosome 5 whereas the intronless genes hDHFR-ψ 2 and hDHFR- ψ4 were assigned to chromosomes 6 and 3 respectively.

9. The intronless gene that perfectly matches the coding sequences of the functional locus (hDHFR- ψ1) was found to be present in the DNA of some individuals and absent from others consistent with a recent evolutionary origin. The presence or absence of this pseudogene represents a novel form of DNA polymorphism.

Significance to Biomedical Research and the Institute Program:

This project is designed to increase our understanding of transcriptional signals that operate at the DNA sequence level in modulating gene expression. The phenomenon of gene amplification, if understood and applied, might have important implications for genetic engineering for eukaryotic cells. Furthermore, the DHFR gene is one of the few available potential dominant selectable markers for use in gene transfer into eukaryotic cells.

Proposed Course of the Project:

The DHFR gene promoter is being characterized by construction of a series of deletion mutants. The intact and deleted promoters are linked to the coding sequences of a gene that encodes an enzyme that confers neomycin resistance. The mutants are also linked to the bacterial gene for chloramphenicol acetyl transferase. Transfection experiments using

these mutants will give a more detailed profile of the DHFR promoter. Preliminary data using these deletion mutants indicate that the minimal promoter requires only 72 bp of upstream sequence. Nonetheless, the functional studies described above suggest that about 500 bp are involved in interaction between protein in a nucleosome free DNaseI hypersensitive region. The nature of these regulatory proteins and the sequences with which they interact are currently under study.

Publications:

1. Chen, M.-J., Shimada, T., Moulton, A.D., Cline, A., Humphries, R.K., Maizel, J., and Nienhuis, A.W.: The functional human dihydrofolate reductase gene. J. Biol. Chem. 259:3933-3943, 1984.

2. Anagnou, N.P., O'Brien, S.J., Shimada, T., Nash, W.G., Chen, M.-J., and Nienhuis, A.W.: Chromosomal organization of the human dihydrofolate reductase genes: Dispersion, selective amplification and polymorphism. Proc. Natl. Acad. Sci. USA. In press.

3. Shimada, T., Chen, M.-J., and Nienhuis, A.W.: A human dihydrofolate reductase intronless pseudogene with an Alu repetitive sequence: Multiple DNA insertions at a single chromosomal site. Gene. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02312 03 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)* Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with β Thalassemia and Sickle Cell Anemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Timothy J. Ley, M.D., Clinical Investigator, CHB, NHLBI
Others: Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
R. Keith Humphries, M.D., Visiting Scientist, CHB, NHLBI
Neal S. Young, M.D., Chief, Section on Cell Biology, CHB, NHLBI
George Dover, M.D., Associate Professor of Pediatrics, Johns Hopkins University, Baltimore, MD.
W. French Anderson, M.D., Branch Chief, MHB, NHLBI

COOPERATING UNITS *(if any)*

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Department of Pediatrics, Johns Hopkins University, Baltimore, MD.

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

5-Azacytidine, a cytidine analog, is capable of causing undermethylation of newly synthesized DNA, and can reactive dormant genes in tissue culture cells and in baboons. This drug has been administered to several patients with β thalassemia and sickle cell anemia; γ globin synthesis was selectively increased in the bone marrow cells of each patient after treatment. The mechanism of augmented fetal hemoglobin production has been under intense investigation during the past year. A study of hemoglobin F production in erythroid progenitor and precursor cells of bone marrow suggests that 5-azacytidine directly alters the globin biosynthetic program in these cells. In addition, study of γ globin gene expression in a hybrid mouse erythroleukemia cells line containing the human 11 chromosome also indicates that the γ globin genes are selectively reactivated by 5-azacytidine in this cell line. These results indicate that 5-azacytidine probably does act, at least in part, by hypomethylating critical control sequences around specific genes.

We have also studied the usefulness of repeated courses of 5-azacytidine in patients with β thalassemia. In two patients, chronic administration of the drug did indeed result in an increased γ globin synthesis, but a decline in β globin synthesis and presumed toxicity to the erythron blunted the clinical response. 5-Azacytidine was not capable of eliminating the transfusion requirement in these patients. Further studies with this drug have been postphoned pending further studies of the effect of other cytotoxic agents on fetal hemoglobin production in humans.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: This study was originally designed to determine whether 5-azacytidine would augment β globin synthesis in humans, and to further understand the mechanisms of globin gene regulation. The globin genes are relatively hypomethylated in tissues where they are highly expressed and completely methylated in normal adult bone marrow. 5-Azacytidine is capable of reactivating repressed genes by causing hypomethylation of newly synthesized DNA. This drug was administered to patients to determine whether hypomethylation of DNA near the genes would be associated with reexpression of these genes. We felt that increased fetal hemoglobin production in patients with thalassemia and sickle cell anemia would provide a new approach to the therapy of these diseases. In addition, the pattern of gene expression in hypomethylated DNA derived from highly differentiated bone marrow cells would yield new insights into the requirements for regulated gene expression.

Methods:

Patients who participated in the 5-Azacytidine trial have had severe β thalassemia or sickle cell anemia associated with severe transfusional hemochromatosis or severe complications of sickle cell anemia, leading to a poor long-term prognosis. Complete laboratory evaluation and control bone marrow studies were performed before 5-azacytidine administration in each patient. 5-azacytidine was then given intravenously or subcutaneously at doses of 1 to 2 kg/day for 3 to 7 days. Laboratory studies and bone marrow examinations were performed to determine the nature of 5-azacytidine effects in these patients.

1. 2×10^7 bone marrow cells are labeled with [^{14}C]leucine in leucine-free IMEM. Lysates of these incubations are analyzed on Triton X - poly acrylamide-urea gals and globin biosynthetic ratios were determined by densitometry tracings of the fluorograms.

2. Bone marrow cells or tissue culture cells were lysed in guanidinium hydrochloride and sarcosyl, and then processed to obtain RNA and DNA. The RNA was analyzed using RNA spot blotting and S_1 nuclease analysis. The DNA was analyzed by restriction endonuclease analysis and Southern blotting techniques in order to determine the frequency of methylated cytosine residues in various regions of total bone marrow DNA.

3. Bone marrow cells obained from treated and normal patients have also been studied in methylcellulose culture. The progeny of different erythroid progenitors (burst forming unit-erythroid or colony forming-unit erythroid) have been evaluated with immunologic techniques to measure the amount of fetal hemoglobin production after treatment. These studies have been performed in collaboration with Dr. George Dover of Johns Hopkins University.

Major Findings:

The effect of 5-azacytidine on erythroid precursor and progenitor cells from bone marrow was studied in 9 patients with sickle cell anemia or severe β thalassemia. Each patient received the drug as an intravenous infusion for 5-7 days. In 8 of the 9 patients, this drug caused a 4-6 fold increase in γ globin messenger RNA concentration in the bone marrow cells. In all 9 patients, the methylation frequency of specific cytosine residues near the γ globin gene promoter was diminished. The percentage of hemoglobin F containing reticulocytes increased within 2 days of starting drug treatment, despite no significant change in the total number of circulating reticulocytes during this period. 5-Azacytidine also acted directly on the late erythroid progenitor cells (colony forming units - erythroid); after 2 days of drug administration, the number of CFU-E that formed colonies in vitro increased in several patients, and these colonies contained an increased amount of hemoglobin F when compared to control colonies. A similar effect was observed in normal bone marrow cells incubated with 5-azacytidine for 24 hours in vitro. The combined direct effects of 5-azacytidine on both the erythroid precursor and progenitor compartments resulted in an increase in hemoglobin F synthesis that was sustained for 2-3 weeks. A general correlation was also noted between the effect of 5-azacytidine on the bone marrow cells incubated in vitro and the hematologic response of individual patients to drug treatment.

In a separate study, we examined the function of the human globin genes in a mouse erythroleukemia cell line (M11 - X) that contained most of human chromosome 11 (the chromosome that includes the human β-like globin gene cluster). In these cells, the human β globin gene was expressed with induction, but the human fetal (γ) and embryonic (ε) globin genes were heavily methylated and repressed. A 24 hour exposure of these cells to 5-azacytidine before induction caused global hypomethylation of DNA, but selective activation of the human γ globin genes. We noted that genomic DNA was remethylated 3-4 days after exposure to 5-azacytidine, but that sequences near the human mouse globin genes remained hypomethylated. This result suggested that the remethylation process was somehow inhibited in the active gene regions, implying that some change had taken place in chromatin structure, or that a nuclear protein had associated itself with these regions. The combined results of these studies suggested that 5-azacytidine was capable of augmenting γ globin gene expression specifically by some alteration in DNA methylation.

Finally, we gave several courses of 5-azacytidine to two patients with homozygous β thalassemia to determine whether augmented γ globin synthesis would improve the efficiency of erythropoiesis and thereby reduce transfusional requirements. Both patients received 5-azacytidine subcutaneously: patient 1 received 2mg/kg/day for 3 days every 2 weeks, (four courses), and patient 2 was treated for 3 days out of 7 for 6 consecutive weeks. At the end of the treatment period, the net increase in non-α-globin mRNA with respect to α globin mRNA was 2.1 fold in patient one, and 4.6 fold in patient two. The absolute reticulocyte

count increased in both patients with treatment, but the hemoglobin concentration actually fell from 11.8 gm/dl to 8 gm/dl in patient one, and from 11.2 gm/dl to 7.5 gm/dl in patient two. When given by these treatment schedules, 5-azacytidine did not modify the transfusional requirements in either patient. The reasons for this lack of success are multifactorial, but probably are related to a decline in β globin synthesis as γ synthesis increases, to cumulative toxicity to the erythron, and to an inability of these patients to tolerate even mild anemia because of significant heart disease.

Significance to Biomedical Research and to Institute Program:

The work described above and previous studies have clearly shown that it is possible to augment fetal hemoglobin synthesis in adults with hemoglobinopathies. This basic finding has spawned a number of other studies designed to evaluate the mechanism of this phenomenon, and to further investigate possibilities for clinical applications. Our basic observations have lent considerable insight into mechanisms of gene regulation in vivo, and have suggested ways by which fetal hemoglobin production might be augmented using other cytotoxic drugs.

Proposed Course of the Project:

During the past year, studies by David Nathan and colleagues (Harvard) and George Stamatoyannopoulos and colleagues (University of Washington, Seattle) have indicated that other cytotoxic agents (e.g. hydroxyurea and Ara-C) are also capable of augmenting fetal hemoglobin production in anemic primates. Since hydroxyurea has essentially no carcinogenic potential, we decided to temporarily halt the use of 5-azacytidine in our patients because of the potential long term risk of developing cancer. In the meantime, careful studies of hydroxyurea in these patients are being performed, (see Individual Project "Pharmacological Manipulation of Fetal Hemoglobin Production in Man," # Z01 HL 02320 01 CHB) and the results compared with previous studies using 5-azacytidine. Again, these comparative studies will add new insights regarding the mechanism of fetal hemoglobin switching, and cellular regulation of hemoglobin production.

Animal studies have been initiated to study the pharmacology, mechanism of action, and toxicity of 5-azacytidine. Eight monkeys received chronic 5-azacytidine administration during the past year, and evaluation of toxicity is ongoing. In addition, we plan to administer a variety of other cytotoxic agents, alone and in combination, to further examine the mechanism of fetal hemoglobin switching, and to continue to try to find practical ways to augment fetal hemoglobin production in humans. We are now employing a new method (F-reticulocyte determination) so that our evaluations of new drugs can be performed much more quickly. Data generated from these studies is currently being assembled in Wylbur, and will soon be subjected to rigorous analyses.

Publications:

1. Ley, T.J., DeSimone, J., Noguchi, C., Turner, P., Schechter, A., Heller, P., and Nienhuis, A.W.: 5-azacytidine increases HbF synthesis and decreases the proportion of dense cells in patients with sickle cell anemia. Blood. 62:370-380, 1983.

2. Ley, T.J., Anagnou, N.P., Turner, P., DeSimone, J., Heller, P., and Nienhuis, A.W.: Globin gene methylation and expression in patients treated with 5-azacytidine. In Globin Gene Expression and Hematopoietic Differentiation. Stamatoyannopoulos, G., and Nienhuis, A.W. (Eds.), Alan R. Liss, New York, 1983, pp. 457-474.

3. Anderson, W.F., Chiang, Y.L., Sanders-Haigh, L., Ley, T.J.: Globin gene expression in somatic cell hybrids. In Globin Gene Expression and Hematopoietic Differentiation. Stamatoyannopoulos, G., and Nienhuis, A.W. (Eds.), Alan R. Liss, New York, 1983, pp. 39-52.

4. Chiang, Y.L., Ley, T.J., Sanders-Haigh, L., and Anderson, W.F.: Human globin gene expression in hybrid 2S MEL x human fibroblast cells. Somatic Cell Genetics. In press.

5. Ley, T.J., Chiang, Y., Haidaris, D., Anagnou, N.P., Wilson, V., and Anderson, W.F.: DNA methylation and regulation of the human β -like globin genes in mouse erythroleukemia cells containing human chromosome 11. Proc. Natl. Acad. Sci. USA, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02313 02 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)* Regulation of Hemoglobin Switching During Development: Characterization of the Human γ Globin Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Stefan Karlsson, M.D., Visiting Associate, CHB, NHLBI

Others: George Keller, M.D., Medical Staff Fellow, CHB, NHLBI
Amanda Cline, Research Assistant, CHB, NHLBI
Austine Moulton, Research Assistant, CHB, NHLBI
Arthur Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to determine whether DNA sequence differences in the promoter regions of the several human globin genes are relevant to their developmental regulation. Our studies have focused on the gamma globin gene promoter. Truncation deletion mutants ranging from 1200 to 55 nucleotides upstream from the start site of transcription have been created by recombinant DNA methods. Linker scanning mutants in which a synthetic linker replaces portions of the promoter have been constructed so as to delete and/or replace conserved sequences within the promoter region. Both deletion and linker scan mutant genes have been introduced into HeLa cells to quantitate their expression. Deletion of increasing amounts of the gamma gene promoter results in progressive decrease in promoter function. In contrast, deletion of one of the two conserved "CCAAT" regions in the promoter results in a 2-6 fold increase in promoter function. The normal gamma and beta globin genes and the various deletion and linker scan mutants of the gamma gene promoter region have been linked to a gene that confers neomycin resistance. These have been introduced into a hematopoietic cell line. Baseline and induced gamma globin gene expression will be measured to determine whether specific DNA regions in the promoter are crucial for its developmental regulation.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives:

The objective of this project is to understand the molecular basis of hemoglobin switching during development. This process is of fundamental clinical importance since activation of the dormant γ globin gene in individuals with severe thalassemia and sickle cell anemia has been shown to be of clinical relevance (see individual project "Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe β Thalassemia and Sickle Cell Anemia"). The questions to be addressed are as follows. First, are the sequence differences demonstrated between the promoters of the individual globin genes relevant to developmental switching? Second are there proteins that interact with these sequences to facilitate transcription of the gene and thereby regulate developmental switching?

Several conserved blocks (boxes) of sequences have been demonstrated to be of functional relevance to β globin gene promoter function. Most well studied is the rabbit β globin gene but the human β globin gene promoter structure is very similar. Thirty bp from the start of transcription is an AT rich sequence that facilitates polymerase binding. Eighty bp upstream is a second conserved block of 5 nucleotides (the CAT box) and beginning at 115 and 100 pairs upstream from the start of transcription are tandemly duplicated 12 bp segments referred to as the CACA box. The γ globin gene promoter is demonstrably different in that the CAT box is duplicated beginning 115 and 80 base pairs from the start of transcription. The CACA box, in contrast, occurs only once beginning 148 bp from the start of transcription. Data has shown that the γ globin gene promoter is enhancer dependent as it is completely nonfunctional in monkey kidney cells without an enhancer whereas addition of an enhancer from the SV40 genome restores promoter function. We have also shown that the CACA box is not absolutely required for promoter function in monkey kidney cells. In contrast, removal of both CAT boxes completely abolishes promoter function. Our present goal is to study specific promoter mutants and hybrid genes transiently introduced into HeLa cells or permanently introduced into MEL and K562 cells in order to obtain a functional profile of the globin gene promoter with special reference to developmental regulation.

Methods:

1. Construction of γ Promoter Mutants. This is accomplished by treating specific restriction endonuclease fragments with the enzyme Bal 31. This enzyme removes both strands of DNA progressively thereby truncating the fragment. The truncated end is "squared" with DNA polymerase I and Bam HI linkers are added by ligation. The truncated fragments are recloned into expression vectors to study their function. An analogous set of upstream deletion mutants is made by beginning the truncation within the promoter region and extending upstream. Complementary mutants are identified by careful sizing of the extent of truncation. Complementary pairs are then reconstructed in the

expression vector. The end result is that one obtains promoter mutants in which the Bam HI linker replaces an equivalent number of nucleotides in the promoter region. Constructs have also been made containing the γ promoter mutant and a neomycin resistance gene in order to be able to permanently introduce the γ promoter mutants into MEL and K562 cells using G418 as a selection agent.

2. Functional Studies of the Mutant Genes: Plasmid expression vectors containing the mutant genes are introduced into HeLa cells in vitro. Forty-eight hours after transfection, the RNA is harvested. Promoter function is quantitated by measuring the relative amount of γ mRNA that beings at the correct initiation site. This measurement is obtained by S_1 nuclease analysis or by primer extension with reverse transcriptase. An intact αglobin gene is introduced as an internal control to allow comparison of γ mRNA to α. mRNA concentration.

3. MEL aprt- cells and semi-sticking K562 cells have been transfected with the γ promoter plasmids and the neomycin resistance gene has also been introduced at the same time, either as a part of the initial plasmid or by using cotransfection of another plasmid that contains the neomycin resistance gene. Single or mixed colonies that are resistant to G418 have been picked and grown up, DNA has been purified and analyzed by Southern blotting using fragments from the promoter and the neomycin resistant gene as probes. The RNA content of these cells is analyzed before or after induction of hemoglobin synthesis, using S_1 nuclease or primer extension analysis.

Major Findings:

1. Truncation mutants that begin 1200, 260, 131, 88, and 55 base pairs upstream from the start site of transcription have been introduced into HeLa cells. The highest level of expression is observed with the -260 truncation. The γ mRNA concentration from this vector is 3 fold higher than the gene having 1200 base pairs of flanking sequence. The gene with progressively shorter promoters show a corresponding decrease in promoter function. For example, the -55 mutant is 10 fold less active than the gene having 260 base pairs upstream from the start site of transcription.

2. Seven different linker scanning mutants have been characterized functionally in the HeLa cell assays. Each shows an increase in transcription over the "wild type" gene. Specifically, replacement of the conserved "CCAAT" box closest to the start site of transcription or its deletion is associated with a several fold increase in transcriptional rate.

3. MEL and K562 cells have been cotransformed with either one plasmid that contains the relevant γ gene mutant and the neomycin resistance gene or two plasmids have been introduced, one containing the γ gene mutant and the other the neomycin resistance gene. In the latter case, the plasmid containing the γ globin gene also includes the

SV40 enhancer. In general, when the two plasmids were introduced at the same time, transformation frequency was higher and the copy number of the genes that were introduced was also higher (10-100) than when using a single plasmid containing both genes.

Significance to Biomedical Research and to the Institute Program.

Patients with transfusion dependent β thalassemia or sickle cell disease could benefit immensely from augmented γ globin chain synthesis and increased formation of HbF. The ability to chronically increase hemoglobin F synthesis might reduce or eliminate the need for transfusions allowing iron stores to be reduced by chelation or phlebotomy. Studies designed to determine the mechanism of hemoglobin switching during development and its regulation are directed toward the long-term objective of obtaining permanent and physiological activation of the human fetal globin gene.

Proposed Course of the Project:

In the near future we will focus on determining whether the duplicated CAT box is important in the developmental regulation of the γ globin gene promoter. This will be done by studying mutants in the CAT box region of the promoter after introduction of the cloned mutants into K562 and MEL cells. Secondly, we will study the possible interplay between promoter sequences and sequences in the gene itself for developmental regulation of the γ gene. In order to do that we will study hybrid γ and β genes in order to determine if there are any sequences in the exons or introns of the β or γ gene that influence expression in stable MEL and K562 transformants.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02314 02 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Function of Proto-Oncogenes in Human Hematopoietic Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

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Medicine Branch, NCI, Section on Cytogenetic Oncology

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects XX (b) Human tissues (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to determine whether proto-oncogenes are functional in human hematopoietic cells and whether these genes are involved in hematopoietic differentiation. The proto-oncogenes are homologous to viral oncogenes; indeed the viral oncogenes have been derived from their cellular homologues. Our studies have focused on the 5 q$^-$ syndrome, a refractory anemia characterized by decreased erythroid precursors and abnormal megakaryocytic differentiation. An interstitial deletion in the long arm of chromosome 5 is characteristically present in such patients. A cellular proto-oncogene (fms) has been localized near the deletion. Using a sensitive S_1 nuclease analysis, we have shown that the fms mRNA is present in bone marrow cells. The protein encoded by the v-fms homologue to the cellular fms proto-oncogene has the characteristics of a membrane receptor. In our studies, we have shown-using monoclonal antibodies raised against the v-fms protein-that the c-fms protein is present on human bone marrow cells. The second facet of this project has focused on the status of the fms proto-oncogene in cells of patients with the 5 q$^-$ syndrome. Bone marrow cells from two of the patients have been fused to CHO cells. In appropriate selective medium, human chromosome 5 is retained in such cells because of the presence of the DHFR locus on this chromosome. Hybrids containing either the normal chromosome 5 or the chromosome 5 having the interstitial deletion have been obtained. In both patients the fms gene has been shown to be deleted from the abnormal chromosome. These data are most consistent with the hypothesis that the fms proto-oncogene encodes for a membrane receptor important for erythropoietic differentiation and that the hemizygosity that results from the 5 q$^-$ deletion effectively reduces receptor number leading to erythroid hypoplasia.

Project Description:

Objectives:

RNA tumor viruses owe their transforming potential to genes acquired by transduction from the genome of animal cells. The transforming genes of retroviruses are called v-oncogenes whereas their cellular homologues from which these are acquired are referred to as proto-oncogenes. Proto-oncogenes may become oncogenic in cells by virtue of mutation; such mutated genes are referred to as c-oncogenes. Many lines of evidence suggest that the proto-oncogenes encode for products that are relevant to cell proliferation and differentiation. One such gene has been shown to encode for the platelet derived growth factor and another for the epidermal growth factor receptor. Therefore, identification of cells in which specific proto-oncogenes are found and analysis of chromosomal rearrangements involving those proto-oncogenes are likely to yield unique insights into cellular differentiation.

One such syndrome is that of refractory anemia and abnormal megakaryocytic differentiation associated with the 5 q^- chromosome. Knowing that the fms proto-oncogene is located near the breakpoint of the deletion, we have designed studies to determine whether the fms proto-oncogene is expressed in bone marrow cells and whether the gene is altered by virue of the chromosomal deletion.

Methods:

1. The bone marrow cells from the patients with 5 q^- syndrome were fused to a $DHFR^-$ CHO cell line. Selective retention of chromosome 5 can be achieved in these hybrid cells using a selective system that requires expression of the DHFR gene. The human DHFR gene is on chromosome 5. The human chromosome complement found in the individual hybrid clones is determined by isozyme and karyotypic analysis.

2. RNA and DNA is prepared from bone marrow cells in patients with the 5 q^- syndrome and from hybrid clones containing either the normal or abnormal chromosome 5. Extraction is accomplished by lysis of cells in guanadinium hydrochloride followed by cesium chloride density centrifugation. Control cells from normal patients, those with erythroid hyperplasia, or various other myelodysplastic syndromes are used for comparison.

3. S_1 nuclease analysis, using genomic DNA fragments from the fms proto-oncogene locus as probes, are used to assay for the presence of fms complementary mRNA in various cell populations.

4. The Southern blotting method is used to analyze DNA from various cells using probes from the fms proto-oncogene locus. DNA is restricted with various restriction endonucleases, resolved by electrophoresis in agarose gels, and transferred to nitrocellulose paper for hybridization to specific probes.

Major Findings:

1. The fms proto-oncogene is expressed in normal human bone marrow cells. Two exons (coding regions) were localized in a 1.6 kb fragment from the 5' end of the fms locus. One of these has been partially sequenced thereby unequivocally establishing the relationship between the complementary mRNA in bone marrow cells, the fms proto-oncogene, and the v-fms sequence previously determined for feline sarcoma virus.

2. Using monoclonal antibodies raised against the v-fms protein, we have shown that the fms proto-oncogene product is expressed on the surface of human bone marrow cells. Preliminary data suggests that the late erythroid progenitor (CFU-E) has the fms protein product on its surface.

3. The fms proto-oncogene has been shown to be missing from the 5 q^- chromosome of two patients with the 5 q^- syndrome. This was accomplished by fusing the bone marrow cells of the patients to the CHO $DHFR^-$ cells. Segregating hybrids containing either the normal or abnormal 5 were analyzed by Southern blot analysis.

4. The bone marrow cells of one of the patients with the 5 q^- syndrome was shown to contain fms rearranged sequences that appeared to segregate independent of chromosome 5. A preliminary interpretation of these data is that a portion of the fms proto-oncogene has been translocated to a different chromosome and therefore segregates in the hybrid cells independently.

Significance to Biomedical Research and the Program of the Institute:

This project is designed to extend the molecular biological techniques to the analysis of hematopoietic differentiation. Probes available from the proto-oncogenes provide an opportunity to achieve this purpose. From these studies we hope to learn about the genes that are specific to particular hematopoietic progenitor populations and to determine how these genes define the subsequent pattern of differentiation of these cells.

Proposed Course of the Project:

Our immediate object is to identify the substance that interacts with the fms proto-oncogene product on the surface of human bone marrow cells. Among the candidates are several growth factors including erythropoietin. Subsequently we hope to define the role that the proto-oncogene product plays in defining the differentiation of specific cell populations. We intend to extend our studies to the analysis of fms proto-oncogene expression in the cells of patients with chronic

myelogenous leukemia. Some patients with this disorder have been identified as having increased fms proto-oncogene expression. We intend to examine the hypothesis that point mutations in the fms proto-oncogene results in increased expression and contributes to the abnormal growth properties of these leukemic cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02315 02 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lymphokines in Aplastic Anemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others: Pedro Gascon, M.D., Medical Fellow, CHB, NHLBI
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Guiseppe Scala, M.D., Visiting Scientist, LMI FCRF NCI
Jeffrey G. Moore, B.A., Chemist, CHB, NHLBI
Eric Raefsky, M.D., Research Associate, CHB, NHLBI

COOPERATING UNITS *(if any)*

Division of Virology, BB and
Laboratory of Molecular Immunoregulation, FCRF, NCI, Frederick, Marvland

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hematopoietic progenitor cells require lymphocytes and their soluble products for normal growth in vitro. Patients with aplastic anemia often respond to anti-thymocyte globulin, a potent immunosuppressive agent. To investigate specific functions of lymphocytes, we have studied lymphokines, the soluble products of these cells, by measuring their production in patients with aplastic anemia and their role in supporting or suppressing bone marrow colony formation. We have identified interferon as the in vitro mediator of hematopoietic suppression in experiments using cells and serum from patients with aplastic anemia. Patients with aplastic anemia have marked abnormalities of interferon production and circulating levels of interferon; the interferon present in the serum from both blood and bone marrow in aplastic anemia is the γ subtype. Normal individuals show small amounts of α interferon in their bone marrow. We have demonstrated marked synergy in the antiproliferative effects of α and γ interferon. Interferon has been demonstrated to directly effect the proliferation of hematopoietic progenitors isolated from primary cell culture. Furthermore, the cell producing interferon in the normal bone marrow is a helper (T4) lymphocyte. In contrast, patients with aplastic anemia show increased numbers of activated suppressor lymphocytes in their peripheral blood (T8 positive, TAC positive, HLADR positive). In at least one patient with aplastic anemia, these cells are responsible for both interferon production and hematopoietic suppression. It appears likely that the mechanism of action of ATG is directed to this abnormal suppressor cell.

Project Description:

Objectives:

Several lines of evidence suggest a role for lymphocytes or their products in the suppression of hematopoiesis characteristic of aplastic anemia. First, occasional bone marrow transplants between syngeneic twins fail in the absence of prior immunosuppressive therapy. Second, anti-thymocyte globulin, a horse serum prepared against human thymocytes and a potent immunosupressive agent, is effective in between 40 and 50% of patients with aplastic anemia. Finally, in vitro studies have demonstrated a role for lymphocytes, possibly in combination with monocytes, in supporting the development of colonies from hematopoietic progenitor cells. Peripheral blood from patients with aplastic anemia may contain lymphocytes that suppress hematopoiesis, and suppressor lymphocytes can be generated from normal peripheral blood by lectin stimulation.

Having identified abnormalities of lymphokine production in aplastic anemia, including decreased production of interleukin 1 by monocytes, increased production of interleukin 2 by lymphocytes, and markedly increased production of interferon by lymphocytes, we have turned to the relationship of these abnormalities to the pathophysiology of bone marrow suppression and aplasia. In addition, the mechanism of action of ATG has been studied, taking advantage of the fortuitous production of a clinically "inactive" batch of anti-thoracic duct lymphocyte globulin in Europe. Active preparations of ATG and ALG have been compared to this inactive lot using a large number of assays.

Methods

Interleukin 1 is obtained by stimulating monocytes with lipopolysaccharide and testing the supernatant for its ability to stimulate mouse thymocyte proliferation. Interleukin 2 is obtained by stimulating mononuclear cells with phytohemagglutinin and testing the supernatant for the ability to support an IL-2 dependent cell line (CT-6). Interferon is measured by its ability to protect several cell lines from virus infection using a plaque assay.

Cell surface markers have been measured using commercially available monoclonal antibodies and peripheral blood mononuclear cells, sheep red blood cell rosetted T cells, and adherent separated monocytes in a fluorescent activated cell sorter (Becton Dickinson FACS-II).

Peripheral blood and bone marrow colonies are measured in methylcellulose using standard techniques and enumeration of colonies by their characteristic morphology; progenitor cells measured include BFU-E, CFU-E (erythroid precursors), CFU-C (granulocyte-macrophage progenitor), and CFU-GEMM (mixed colony-progenitor).

Suppressor cells are generated by stimulation of peripheral blood T cells with lectins such as pokeweed mitogen, phytohemagglutinin, and concanavalin A and assayed in colony culture systems.

ATG and ALG have been assayed using chromatography in cellulose for immunoglobulin content; polyacrylamide gel electrophoresis for preparations of immunoprecipitates of ATG/ALG and thymocyte or lymphocyte cell membranes for binding specificity; fluorescent activated cell sorting using lymphocytes, granulocytes, and platelets for tissue specificity; ability to stimulate interleukin 2 and hematopoietin production; and direct cytotoxicity with ^{51} labelled lymphocytes.

Results:

Lymphokines in Patients with Aplastic Anemia: Circulating interleukins 1 and 2 cannot be measured directly in the serum, but interleukin production by stimulated cells from the blood may be quantitated. Peripheral blood mononuclear cells from patients with aplastic anemia show markedly decreased interleukin 1 production and very high interleukin 2 production. The interleukin 2 receptor, as determined by fluorescent activated cell sorting analysis using the anti-Tac antibody, is expressed at high levels on the cells of patients with bone marrow aplasia. These abnormalities are not present in patients with pancytopenia due to bone marrow processes, nor do they occur in patients treated with cytotoxic drugs to bone marrow aplasia. These results suggest that the lymphocytes of patients with aplastic anemia are primed for interleukin 2 production, possibly because of decreased levels of circulating interleukin 2 and the absence of the required interleukin 1 stimulation of lymphocytes.

Increased interferon production and high blood and bone marrow serum levels of interferon are restricted to patients with aplastic anemia and are not present in patients with induced bone marrow failure or pancytopenia due to cellular bone marrow diseases. The subtype of interferon present in blood and bone marrow has been determined, using blocking experiments with highly specific monoclonal antibodies and specific cell targets in the interferon assay. The bone marrow interferon present in aplastic anemia patients consists of both alpha and gamma subtypes, in contrast to the alpha interferon species present in patients with other diseases like acquired immunodeficiency syndrome and systemic lupus erythematosus. As alpha and gamma interferons have synergy in their antiproliferative effects on hematopoietic colony formation, the absence of gamma interferon in diseases other than aplastic anemia are consistent with a role for interferon in bone marrow suppression in aplastic anemia.

Suppressor Cells in Aplastic Anemia: The presence of activated suppressor cells in aplastic anemia has been determined using two color flow microfluorometry. By this method, human cells have been labelled with two antibodies, each bearing a separate fluorochrome, to allow the

detection of multiple antigens simultaneously on cell populations. In aplastic anemia, there is a marked increase in the number of suppressor (T8 or Leu 2) lymphocytes that bear either the Tac or HLA-DR antigens. Both in vitro and in vivo, the presence of these antigens on lymphocyte cell surfaces is associated with cell activation, for example following lectin or antigenic stimulation. In addition, we have determined that the same cells that bear Tac also bear the HLA-DR antigen. Furthermore, the presence of activated lymphocytes has been related to interferon production. In normal peripheral blood and bone marrow, Tac positive cells produce interferon. Similarly, in aplastic anemia patients, the Tac positive cell in the peripheral blood and bone marrow produces interferon that also effects hematopoietic suppression. In normal individuals, the helper lymphocyte bearing Tac antigen is the interferon producing cell following in vitro lectin stimulation, whereas in patients with aplastic anemia the activated suppressor lymphocyte spontaneously produces interferon. Probably the suppressor lymphocyte in bone marrow failure produces the gamma interferon that may cause hematopoietic suppression.

Mechanism of Action of ATG: Anti-thymocyte globulin (ATG) is produced by immunizing horses with human cells. ATG is not specific for human T cells, however, and binds well to all circulating peripheral blood cells as well as to bone marrow cells, thymus, and testis cell membranes. ATG also binds to nuclear and cytoplasmic components of visceral tissues. ATG contains measurable quantities of interleukin 2, and ATG is a potent mitogenic agent for human lymphocytes and promotes their production of interleukin 2. T cells conditioned by exposure to ATG produce media that is rich in hematopoietins, consistent with the coordinated expression of both lymphokines and hematopoietins by T cells. Together, these results suggest that one mechanism of action of ATG may be by providing interleukin 2 and other growth factors to patients who are deficient in production of hematopoietins; the endogenous lymphokine production they stimulate is then adequate for continued growth factor production.

The Swiss Serum Institute has recently produced a preparation of horse antithoracic duct lymphocyte globulin (ALG) that has been inactive in clinical trials in patients with aplastic anemia. We have analyzed this preparation in comparison to active preparations of ALG and ATG. All preparations contain exclusively immunoglobulin of the IgG type in approximately equivalent concentrations. The ability of these preparations to stimulate lymphocyte blastogenesis and hematopoietin production are also equivalent. No consistent differences have been discovered in the binding specificity of these preparations to lymphocytes, granulocytes, and platelets. However, there is a significantly poorer ability of the inactive preparation to cause complement-mediated lysis of human T cells. Attempts to immunoprecipitate specific antigen with the active preparations not immunoprecipitated by the inactive preparations have so far been unsuccessful. These results are most consistent with a cytotoxic effect of ATG/ALG on a suppressor cell, perhaps bearing on unusual antigen because it is activated.

Significance to Biomedical Research and to the Program of the Institute:

Determination of the mechanism of action of ATG may permit development of more effective and less toxic drugs for the treatment of aplastic anemia, such as purified growth factors or selective antisera or monoclonal antibodies. Furthermore, these studies bear on the role of lymphokines in normal bone marrow development and their role in immunological reactions in general.

The description of interferon as the effector of hematopoietic suppression and the identification of a specific interferon producing cell, the activated suppressor lymphocyte, have implications in the study and treatment of aplastic anemia. First, the identification of a specific lymphokine and the specific cell using phenotypic markers provides a more reliable and simpler methodology for the study of hematopoietic suppression. Second, advantage should be taken of parallels between the regulation of hematopoietic cell proliferation and regulation within the immune system. Finally, it appears likely that the mechanism of action of antithymocyte globulin in the majority of patients with aplastic anemia in whom this treatment is effective is by destruction of the activated suppressor cell. The most promising theoretical consideration is that the ATG preparations recognize specific antigens present on both the original immunogen and on these activated cells, destroys them, and leads to reduction in interferon production.

Proposed Course of the Project:

The origin of high circulating levels of interferon and the reasons for high production of interferon in vitro will be investigated by determining interferon types and investigating the potential role of chronic viral infections in stimulating interferon production (see Individual Project: Hematopoiesis in Bone Marrow Failure). Interferon's effects on lymphocyte subpopulations will be investigated by in vitro stimulation studies and FACS measurements.

Alterations in lymphokine abnormalities are being analyzed prospectively in patients who are treated with antithymocyte globulin and correlated with hematopoietic response. Precise mechanism of action of gamma interferon at the hematopoietic cell surface and its ultimate functional role in regulating the cell cycle are being analyzed with in vitro experiments using normal and aplastic blood and bone marrow cells. Attempts to identify the active agent within antithymocyte globulin preparations are being pursued with detailed cytotoxicity studies and Western gel blotting.

Publications:

1. Zoumbos, N.C., Ferris, W.O., Hus, Su-Ming, Goodman, S., Griffith, P., Sharrow, S.O., Humphries, R.K., Nienhuis, A.W., and Young, N.S.: Analysis of lymphocyte subsets in patients with aplastic anemia. Brit. J. Haemat. In press. 1984.

2. Zoumbos, N., Gascon, P., and Young, N.S.: The function of lymphocytes in normal and suppressed hematopoiesis. Blut. 48:1-9, 1984.

3. Gascon, P., Zoumbos, N.C., and Young, N.S.: Immunological abnormalities in patients receiving multiple blood transfusions. Ann. Int. Med. 100:173-177, 1984.

4. Young, N.S. and Speck, B.: Anti-thymocyte and anti-lymphocyte globulins: Clinical trials and mechanism of action. In, "Aplastic Anemia: Stem Cell Biology and New Clinical Concepts." (Eds: Young, N., Levine, A., and Humphries, R.K.) Alan R. Liss, New York, pages 1-12, 1984.

5. Humphries, R.K., and Young, N.S.:. Aplastic anemia and stem cell biology. In "Aplastic Anemia: Stem Cell Biology and New Clinical Concepts." (Eds: Young, N., Levine, A., and Humphries, R.K.) Alan R. Liss, New York, pages 129-139, 1984.

6. Zoumbos, N., Djeu, J., and Young, N.: Interferon is the mediator of lectin induced suppression of hematopoiesis. J. Immunol. In press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02316 02 CHB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Serum Sickness Following ATG Treatment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: Neal S. Young, M.D., Chief, Section on Cell Biology, CHB, NHLBI
Thomas Lawley, M.D., Senior Investigator, D, NCI
Kim Yancey, M.D., Senior Investigator, D, NCI
Michael Frank, M.D., Chief, LCI, NIAID
Arthur Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS (if any)

Dermatology Branch, NCI; Laboratory of Clinical Investigation, NIAID

LAB/BRANCH

Clinical Hematology Branch

SECTION

Section on Cell Biology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN YEARS	PROFESSIONAL	OTHER
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

X (a) Human subjects
X (a1) Minors
(a2) Interviews

X (b) Human tissues

(c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the development of serum sickness in humans with the use of various in-depth clinical and immunological analyses. Thirty-five (35) patients with various hematological dyscrasias treated with horse anti-thymocyte globulin (ATG) infused over a 10 or 28 day course have been evaluated. The majority of patients had aplastic anemia (29/35). Thirty (30/35) patients developed clinical signs and symptoms of serum sickness 8-14 days after the start of ATG infusion that consisted of fever/malaise (94%), rashes (80%), arthralgias (66%), gastrointestinal complaints (57%), cephalgias (49%), myalgias (31%), blurred vision (31%), and lymphadenopathy (14%). We have also discovered a peculiar cutaneous marker occuring on the hands and feet that is specific for serum sickness. The formation of immune complexes and the consumption of complement paralleled the development of the serum sickness symptoms. Immunoglobulin levels increased several-fold in patients developing serum sickness with the most dramatic increases in the immunoglobulin associated with allergic diseases, IgE. Abnormalities in renal and liver function were noted during serum sickness. Acute phase reactants as measured by erythrocyte sedimentation rates, titers of C-reactive proteins, fibronectin and beta-2 microglobulin became markedly elevated during the course of infusion of ATG. Hematopoietic recovery occurred in all patients that did not develop serum sickness as compared to a 50% recovery rate in those that did develop serum sickness.

Project Description:

Objectives: The objectives of these studies are to correlate serum sickness indices to hematopoietic recovery and delineate the human immune response to heterologous antigen using modern assays.

Methods:

The patient population included in these studies include those patients assigned to receive ATG for their hematological disorder.

1. Immune complexes are measured with the Clq and Raji cell assays.

2. Complement levels C3 and C4 are measured with an hemolytic assay.

3. Clinical symptoms are monitored daily for routine patient care. These include dermatological biopsies for evaluation of dermatoses and ophthalmology consults for problems with vision.

4. Liver function tests, urinalyses, renal function tests, acute phase reactants and immunoglobulins are routinely monitored.

Major Findings:

1. Serum sickness symptoms directly correlate to formation of immune complexes and consumption of complement. Complement levels drop immediately with the first infusion of ATG and then again at the height of clinical serum sickness. Immune complex formation does not usually begin until day 11±2.

2. The skin manifestations appear to have a distinct appearance in the webs of the fingers and the soles of the feet. This sign has appeared in 70% of patients developing serum sickness and appears to be a specific marker for serum sickness. Biopsies have revealed non-distinct mild inflammatory changes with blood extravasation.

3. The eye manifestations are unusual transient retinal and conjunctival hemorrhages.

4. The appearance of serum sickness decreases the chance of hematopoietic recovery by 50%.

5. IgE antibody specific for the horse protein appears at various times during the course of clinical serum sickness.

6. Abnormalities in renal and liver function are also noted during serum sickness in 20% of patients that develop serum sickness.

Proposed Course of the Project:

We intend to evaluate the immune response in these 35 patients to delineate the differences between those that responded to treatment versus those that did not respond. Other indices to be evaluated are the types and components of the immune complexes formed, skin tests, the clearance of radioactively labelled ATG and the reticuloendothelial clearances of antigen. We intend to complete this evaluation over the next year.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02317 02 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Expression of Glycoprotein Hormone Genes in Pituitary and in Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Stephen S. Lippman, M.D., Ph.D., Medical Staff Fellow, CHB, NHLBI
Others: Bruce D. Weintraub, M.D., Senior Investigator, MC, NIADDK
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
Steven Nagelberg, M.D., MCR, NIADDK
Joan Burnside, Ph.D., MCR, NIADDK

COOPERATING UNITS *(if any)*

Molecular and Cellular Regulation Section (MCR), NIADDK

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glycoprotein hormones (TSH, LH, FSH, CG) each contain a common α subunit combined with a unique β subunit to form the active hormone. The α and β subunits are homologous and the α subunit is always present in molar excess in normal pituitary. This is analogous to hemoglobin synthesis which requires the coordinate expression of the α globin gene with an additional homologous gene (β, γ, δ, ε) to produce a functional hemoglobin molecule.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives:

1. To determine the primary site of regulation of glycoprotein hormone synthesis (transcriptional, translational, or post-translation).

2. To demonstrate that the immunoreactive glycoprotein hormone-like material detected in some tumor lines is indeed synthesized by these cells, by demonstrating the presence of the specific mRNA for glycoprotein hormone subunits.

3. To demonstrate whether the ectopic production of glycoprotein hormones by tumors is an example of activation of a cellular "oncogene" as has been recently demonstrated for PDGF, or whether ectopic production is actually ectopic expression of the normal gene.

Methods:

1. Isolation of RNA and DNA from tumor cells in culture, pituitary tumors passaged in animals, and from normal pituitary (mouse) using cell lysis in Guanidine Thiocyamate and CsCl gradients or Lithium Chloride precipitation.

2. Characterization of mRNA:

a) using glyoxal agarose gels and Northern blotting with nick-translated full-length cDNA probes (HCG and TSH).

b) primer extension mapping using end-labelled, synthetic oligonucleotide probes.

3. Restriction fragment mapping of tumor glycoprotein hormone genes using Southern transfers and hybridization with nick-translated cDNA probes.

4. Transfection of ectopic glycoprotein hormone producing tumor DNA into NIH 3T3 cells.

Results:

Intact mRNA as judged by the integrity of 18S and 28S ribosomal RNA on denaturing glyoxal gels has been isolated by the Guanidinium Thiocyamate lysis - Lithium Chloride precipitation technique from human term and preterm placental tissues, from mouse pituitary and thyrotropic tumor, and from several cell lines including JEG, a trophoblastic carcinoma, HeLa, a cervical carcinoma, and A172, a glioblastoma.

Integrity of specific mRNA for HCG alpha and beta has been confirmed by Northern blot analysis of denaturing glyoxal agarose gels of placental and JEG total RNA preparations.

Ectopic expression of HCG alpha but not beta mRNA has been detected by Northern blot analysis. Since under the hybridization conditions used, the full length cDNA probes hybridized only to their specific mRNAs as confirmed by Northern blot analysis, we have been able to set up a quantitative Dot blot assay for both HCG alpha and beta mRNA using total RNA from preterm human placental RNA as standard.

Preliminary results show at least a 10x accumulation of alpha and beta mRNA 48 hours after stimulation of JEG cells in culture with 8 bromocyclic AMP. The alpha mRNA level increases rapidly and can be detected at 2 hours while the increase in beta mRNA level is only detectable at 48 hours.

Primer extension of a short synthetic DNA oligomer complementary to HCG alpha mRNA shows a single and identical 5' end for mRNA from placenta and from JEG cells. Primer extension of a short synthetic DNA oligomer complementary to HCG beta mRNA shows multiple 5' terminae in placental mRNA in the same preparation of RNA. Only a single predominant terminus is seen in beta mRNA from JEG cells.

Primer extension of short synthetic DNA oligomers end-labelled with ^{32}P is sensitive enough to detect the predicted 5' terminus for mouse TSH alpha mRNA in RNA isolated from a single mouse pituitary; however, a similar oligomer complementary to mouse TSH beta mRNA gives multiple terminae the identity of which has not been confirmed. Further work in this system will be done with full length cDNA probes obtained from Dr. William Chin (Howard Hughes Medical Research Institute).

Significance to Biomedical Research and to the Program of the Institute:

The collaborative nature of this project provides an opportunity to simultaneously analyze the full complexity of the coordinate expression of the gene products from the genomic to post-translation levels.

Proposed Course of the Project:

Project is to continue in the Molecular and Cellular Regulation Section (MCR), NIADDK with the Clinical Hematology Branch, NHLBI as a cooperating unit.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02318 01 CHB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enhancer and Promoter Specificity of Immunoglobulin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Takashi Shimada, Visiting Fellow, CHB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tissue-specific enhancers seem to play a major role in controlling developmentally regulated gene expression. Expression of rearranged immunoglobulin genes introduced into both lymphoid and non-lymphoid cells has led to the identificiation of tissue-specific transcriptional enhancer sequences in the major intron between the J and C region of the immunoglobulin gene. We have shown that immunoglobulin gene expression may also be controlled by tissue-specificity of the promoter. The K chain V region promoter contain sequences at the 5' side of the promoter which seems to inhibit promoter activity in non-lymphoid cells. Tissue-specificity of immunoglobulin gene enhancers could be due to its interaction with some, as yet unidentified, trans-acting regulatory factors that may be present only in lymphoid cells. We have constructed special plasmid vectors containing the coding sequences of a dominant selectable marker gene that confers resistance to neomycin. These coding sequences are driven by an immunoglobulin gene promoter and the various vectors contain different immunoglobulin gene enhancers. By introducing these hybrid genes, we hope to create recipient cells that can be used to directly isolate genes that code for putative trans-acting regulatory factors. Isolation of these regulatory genes will greatly enhance our ability to understand the regulation of tissue-specific gene expression at the molecular level.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives and Methods:

The main objective of this study is to investigate the mechanism(s) of tissue-specific gene regulation. Introduction of rearranged immunoglobulin gene clones (both heavy and light chain) into a variety of cell types has shown the presence of "enhancer" elements in the intron between the J and C region of the gene. They are necessary for the expression of immunoglobulin genes in lymphoid cells. These results suggested the presence of lymphoid cell specific factors that may interact with the enhancer region of immunoglobulin genes to promote their exclusive expression in immunoglobulin producing cells. To study the role of such specific enhancer recognizing factors (SERF) in regulating tissue-specific gene expression, we devised a scheme-involving gene transfer and recombinant DNA methodologies to directly isolate genes that code for such putative regulatory factors. Once the regulatory gene becomes available, it could be used to answer specific questions relating to the organization and interaction between structural and regulatory genes at the molecular level. The knowledge gained through these studies may be applied to other systems such as the developmental regulation of globin gene expression.

Methods:

We constructed plasmid vector designed to facilitate isolation of the gene coding for the putative SERF gene using a gene transfer method. Included in the vector were: (1) the coding sequences for a dominant selectable marker; (2) a promoter that is stringently dependent upon an enhancer for its function; and (3) tissue specific enhancer elements from various immunoglobulin genes.

As the dominant selectable marker we chose the bacterial "Neo" gene that has been used successfully in this laboratory. After testing promoters of different genes, the mouse κ chain immunoglobulin gene promoter was chosen because it was totally inactive without an enhancer (we used the enhancer from Harvey sarcoma virus LTR region to test the enhancer dependency of promoters). Enhancer regions from mouse κ light chain, u heavy chain and human κ light chain immunoglobulin genes were chosen to insert into the test vector. These fragments were linked appropriately and the constructions were completed using standard cloning vectors. The coding sequences that confer neomycin resistance were replaced with those for the bacterial gene for chloramphenicol acetyl transferase (CAT) to create vectors that could be used to study function of the immunoglobulin promoter-enhancer combination using a transient gene expression assay.

The next step was to determine whether these test gene vectors demonstrated tissue specificity as reflected by lack of function in non-lymphoid cells but function in myeloma cells. If this condition was satisfied, then the strategy is to isolate clones of mouse L cells (a non-lymphoid cell line) having the Neo test gene vector stably

integrated in a non-expressible manner followed by its activation by transfection with mouse or human myeloma DNA. It would then be possible to clone the SERF gene using conventional methods.

Major Findings:

The Neo test gene vectors when introduced into mouse L cells did not give rise to any Neo resistant colonies. These vectors are currently being tested for their function in myeloma cells. The results were somewhat different when the same vectors containing the CAT gene instead of the Neo gene were tested for transient expression in different non-lymphoid cells, mouse L cells, 3T3 cells and HeLa cells. In 3T3 and HeLa cells none of these vectors containing either mouse κ, human κ or mouse μ chain enhancers were active in driving the CAT gene whereas in mouse L cells both K chain enhancers and but not the μ chain enhancer were active in enhancing the CAT gene expression. The CAT gene vectors are also being tested in mouse myeloma cells.

Inhibitory sequences have been identified 5' to the mouse immunoglobulin κchain V region promoter. While constructing the various Neo gene vectors described above, the κ chain promoter region containing either 250 bp or 650 bp was used. The shorter promoter, totally inactive in driving the Neo gene in L cells, was fully active when placed under the influence of Harvey sarcoma virus enhancer. On the other hand, the longer 650 bp promoter was totally inactive in L cells even with Harvey enhancer. Analogous results were obtained in transient assays using the CAT gene vectors. This region may bind to a negative regulatory factor that may be present only in non-lymphoid cells in order to keep this gene totally inactive. Such negative factors may not be present in non-lymphoid cells.

We have also isolated a number of clones of L cells carrying the Neo gene in test vectors. These cells do not express the neo gene since they will not grow in neomycin. These clones are being analyzed by Southern blotting to ensure that the Neo gene with immunoglobulin promoter and enhancer are integrated without any rearrangement.

Significance to Biomedical Research and to Institute Program:

This project is designed to investigate the regulation of tissue-specific genes at the molecular level. In addition to its fundamental biological significance, understanding control of developmentally regulated gene expression may have clinical relevance in regard to the pathogenesis of malformations including tumoregenesis due to unscheduled expression of developmentally regulated cellular oncogenes.

Proposed Course of the Project:

We have isolated several mouse L cell clones with the Neo test gene vectors integrated stably but in a non-expressible manner. The immediate goal is to analyze these clones by Southern blotting to verify the physical integrity of the intergrated Neo gene vectors. Clones that contain the intact gene without rearrrangement will be used for further analysis. DNA from mouse and human myeloma cells will be used for transfection into these L cell clones followed by selection for neomycin resistance. Development of neomycin resistance in transfected colonies would indicate the activation of the dormant Neo gene by some product, perhaps the putative SERF, coded by myeloma cell DNA. DNA from Neomycin resistant primary clones arising from transfections using human DNA will be used to isolate secondary transformants by repeat transfection. The DNA from these cells can then be used to construct a bacteriophage or cosmid library for screening with a human repetitive sequences "Alu" probe. We hope to isolate the gene responsible for activation of the neomycin resistance gene by interaction of its product with the immunoglobulin gene promoter or enhancer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02319 01 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Viruses and Bone Marrow Failure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Eric Raefsky, M.D., Medical Staff Fellow, CHB, NHLBI

Others: Neal S. Young, M.D., Chief, Section on Cell Biology, CHB, NHLBI
Leonidas Platanias, M.D., Visiting Fellow, CHB, NHLBI
Maria Harrison, Research Assistant, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to study the mechanism of retroviral induced aplastic anemia and to ascertain if some cases of human aplastic anemia are associated with a persistent retroviral infection. Though feline leukemia virus (FeLV) is well known to cause leukemias in cats, it actually causes more cases of erythroid hypoplasia and aplastic anemia. Therefore, the pathogenesis of FeLV induced aplastic anemia will be studied in order to gain insights into the development and etiology of human aplastic anemia. In addition cells from the bone marrow and peripheral blood from patients with aplastic anemia will be grown in culture in an attempt to induce retroviral growth.

4 5-0

Project Description:

Objectives:

The etiology of aplastic anemia is unknown in the majority of cases. However, many lines of evidence suggest that this disease may be secondary to a persistent retroviral infection in at least a proportion of cases. The lymphocytes of patients with aplastic anemia have dysregulated production of lymphokines such as interferon and interleukin II. This has also been found in lymphocytes infected with feline leukemia virus (FeLV) or human T cell leukemia virus (HTLV). These lymphocytes also have abnormally elevated expression of the Tac antigen, the receptor for interleukin II. This has been found in only one other disease, namely adult T cell leukemia (ATL) which is due to HTLV infection. T cells from patients with ATL have elevated expression of the HLA-Dr molecule which is a marker of T cell activation that is elevated during viral infections. Finally, retroviral infection is a common cause of aplastic anemia in animals. In particular, although FeLV is well known to cause lymphosarcomas and leukemias in infected cats, it more commonly causes hematodepressive diseases such as erythroid hypoplasia or frank aplastic anemia. Thus, the object of this project is to study the pathogenesis of FeLV induced aplastic anemia and to ascertain if some cases of human aplastic anemia are associated with a retroviral infection.

The following questions concerning FeLV induced aplastic anemia will be studied by our group over the next several months. Is the occurrence of aplastic anemia virus strain specific or is a virus isolated from a FeLV infected, pancytopenic cat able to cause different disease when innoculated into different cats? Does *in vitro* infection of hematopoietic progenitors in either short term or long term bone marrow cultures with different FeLV strains result in specific effects on different cell lineages, and do these differential effects mimic the *in vivo* status of the cat from which the virus was isolated? Do FeLV-negative, anemic or pancytopenic cats harbor latent FeLV as has been shown for FeLV-negative cats with lymphomas. What are the differences on a molecular level between various FeLV strains which account for their differential effects? Finally, what are the molecular events in FeLV infections that results in a degenerative versus a proliferative response?

We will also grow both bone marrow and peripheral blood mononuclear cells from human patients with aplastic anemia in culture in an attempt to induce retroviral growth. Testing for the presence of virus will be done in two ways. First, we will directly look for viruses using electron microscopy. Second, we will assay the culture supernatant for the presence of RNA dependent DNA polymerase (i.e., reverse transcriptase), an enzyme unique to retroviruses. If a virus is isolated, more detailed molecular analysis is planned.

Methods:

Feline bone marrow is cultured in methylcellulose to allow growth and enumeration of colonies which represent various hematopoietic progenitor cells. These include BFU-E (the early erythroid precursor), CFU-E (the late erythroid precursor), and CFU-C (a granulocyte-macrophage precursor). In vitro infection with different strains of Felv is carried out under various protocols to determine optimal conditions.

Bone marrow and peripheral blood mononuclear cells from patients with aplastic anemia are grown in long term cultures in an attempt to induce retroviral growth. Peripheral blood cells are initially stimulated with PHA and are then maintained in media containing fetal calf serum and partially purified T cell growth factor (TCGF). Bone marrow mononuclear cells are processed in an identical fashion. In selected cases, bone marrow cells are also grown in a more classic long term culture system with media containing fetal calf serum, horse serum, and hydrocortisone. Cells are removed twice weekly for electron microscopy and the supernatants are processed for reverse transcriptase activity. This assay measures the incorporation of tritiated thymidine triphosphate on an oligo.dT poly rA template-primer in the presence of either magnesium or maganese. To test for contamination with DNA polymerase, these samples are also assayed with an oligo.dT poly dA template primer.

Major Findings:

Pathogenesis of FeLV induced aplastic anemia: In order to study the effects of FeLV on hematopoiesis, an in vitro feline hematopoietic colony assay was developed. The time course of colony formation was studied and optimal timing for colony enumeration was determined. The reagents needed for colony growth and their optimal concentrations was also determined. A linear relationship between the number of cells plated and the number of colonies formed was found. In addition, reliable methods for growing and titering FeLV were ascertained. The in vitro infection of feline bone marrow cells by different FeLV strains has only recently begun and no definitive results have yet been found.

Cultures of blood and bone marrow from human patients with aplastic anemia for retroviral growth: This project has also just begun. Samples from both patients with aplastic anemia and normal volunteers as controls have been obtained for electron microscopy and reverse transcriptase activity. These results are pending at this time.

Significance to Biomedical Research and to the Program of the Institute:

The understanding of the pathogenesis of FeLV induced aplastic anemia may lead to significant insights into the etiology and development of human aplastic anemia. The finding of an association between some cases of human aplastic anemia and a chronic retroviral infection would considerably advance our understanding of the cause of aplastic anemia and may permit more specific and less toxic treatment regimens to be devised.

Proposed Course of the Project:

As this project has recently started, no definitive results have yet been obtained and the course of the project is well outlined above.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02320 01 CHB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacological Manipulation of HbF Synthesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

Others: George Dover, M.D., Department of Pediatrics, Johns Hopkins University
Neal S. Young, M.D., Chief, Section on Cell Biology, CHB, NHLBI
Jeffrey Moore, Chemist, CHB, NHLBI
Timothy J. Ley, M.D., Senior Investigator, CHB, NHLBI

COOPERATING UNITS *(if any)*

Department of Pediatrics, Johns Hopkins University, Baltimore, Maryland

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies are designed to determine whether hydroxyurea, a cytotoxic agent, will cause significant production of HbF in patients with sickle cell anemia. Studies in experimental animals have shown modest but significant increases in HbF synthesis after drug treatment. We have administered the drug in varying doses and at different intervals to ten patients with sickle cell anemia. High doses employed initially (50mg/kg) caused significant hematopoietic toxicity in some patients. Analysis of peak drug levels and the rate of clearance following a single oral dose uncovered significant heterogenity among patients; marrow depression occured in patients with the highest levels and the slowest rate of clearance. Subsequent readjustment of the dose based on pharmacokinetic measurements allowed us to avoid toxicity. The most effective regimen employed to date is the administration of 40-50mg/kg once per week. On this schedule, several patients have exhibited a progressive increase in HbF synthesis to 6-12%. Two additional patients with severe beta thalassemia have been treated. Once exhibited a modest response with a reticulocytosis and stabilization of the Hb concentration.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives:

Patients with severe beta thalassemia or sickle cell anemia could significantly benefit by increased HbF production. In thalassemic individuals, synthesis of the gamma chain (found in HbF) compensates for the deficiency of beta chain production. The imbalance in globin synthesis, characteristic of this syndrome, is partially corrected by increased gamma globin production. Reduction of the intracellular HbS concentration by equal molar replacement with HbF reduces the polymerization potential of intracellular hemoglobin, and therefore the sickling propensity of SS cells.

Our initial studies showed that 5-azacytidine was capable of increasing HbF production in humans with these disorders. Two mechanisms seemed possible. The first involves DNA hypomethylation that is characteristic of rapidly proliferative cells exposed to 5-azacytidine. Expressed genes, including the gamma globin gene, are characteristically undermethylated compared to their status in cells in which these genes are silent. The second hypothesis to account for the mechanism of action of 5-azacytidine is that it perturbs stem cell kinetics, presumably by a cytotoxic effect. Based on this hypothesis, other investigators administered hydroxyurea to primates and were able to show increase in HbF synthesis.

Because hydroxyurea appears to have little if any carcinogenic potential, we have chosen to test its efficacy in sickle cell anemia patients and have deferred any further use of 5-azacytidine. Our studies show that the drug is active, although the mechanism by which increased HbF synthesis is achieved differs in several respects from that of 5-azacytidine.

Methods:

1. Hydroxyurea is administered to patients at various doses and with varying intervals.

2. The blood level of hydroxyurea has been measured in several patients following drug administration in collaboration with Dr. George Dover.

3. The hematological response to hydroxyurea is monitored by measuring the percentage of reticulocytes that contain HbF (F-reticulocytes) by radial immunoprecipitate formation. The amount of HbF per F-reticulocyte was quantitated by measuring the diameter of the immunoprecipitate. Standard hematological measurements include complete blood count, reticulocyte count, and HbF.

4. Colony formation was monitored in cultures of bone marrow obtained before, during and following hydroxyurea administration. The cultures were established in methylcellulose in the presence of hematopoietic growth factors under standard conditions. The amount of HbF per F-nucleated red cell in individual colonies was assayed immunologically.

Major Findings:

1. The occurrence of significant bone marrow depression in several patients treated initially prompted a careful examination of the pharmacokinetics of hydroxyurea. The dose recommended based on animal studies (50mg/kg/day for 5 days) was shown to cause highly variable blood levels of the drug. Eighty percent of hydroxyurea is excreted unchanged in the urine. A direct correlation was found between the serum creatinine level and an indirect correlation with creatinine clearance and the peak drug levels.

2. Adjustment of the dose based on these pharmacokinetic measurements led to successful stimulation of F-reticulocyte formation in several patients. The amount of HbF per F-reticulocyte was not changed by hydroxyurea treatment. Administration of a single dose weekly for several weeks to two patients resulted in a progressive increase in HbF in peripheral blood. One patient whose HbF was less than 1% before treatment reached a level of 6% after six weeks of drug administration. A second patient began at a level of 2.7% and reached a level of 12% after six weeks.

3. Hydroxyurea appears to be highly toxic to erythroid progenitors. Colony number was reduced drastically following drug administration. No significant increase in HbF per F-nucleated red cell was observed in those colonies that formed following hydroxyurea administration compared to control colonies.

4. Two patients with homozygous beta thalassemia received hydroxyurea in doses capable of stimulating F-reticulocyte formation in patients with sickle cell anemia. There was no increase in the amount of HbF per F-reticulocyte and also drug administration did not change the ratio of gamma to beta mRNA in bone marrow. Nonetheless, one of the patients exhibited a modest increase in reticulocytes and his hemoglobin concentration stabilized for two weeks. These data seem most consistent with a mechanism of action of hydroxyurea by which early erythroid precursors, usually destined for cell death because of the marked imbalance of globin biosynthesis, are salvaged because of a modest increase in gamma globin production.

5-Azacytidine causes an increase in F-reticulocyte number, an increase in HbF per F-reticulocyte, and a dramatic increase in gamma

mRNA concentration in bone marrow cells of thalassemic patients. In addition, the number of late erythroid progenitors that form colonies in vitro are increased early in the course of 5-azacytidine administration. In contrast, hydroxyurea does not increase HbF per F-reticulocyte, it kills hematopoietic progenitors, and does not lead to a detectable increase in gamma mRNA in the bone marrow cells of thalassemic patients. These observations suggest a significantly different mechanism of action for the two drugs.

Significance to Biomedical Research and to Institute Program:

Sickle cell anemia and beta thalassemia are severe diseases that cause serious morbidity and mortality. The developmentally silenced but structurally normal gamma globin gene, if activated, could compensate for the genetic defects affecting the beta globin gene in these conditions. Furthermore, these studies (and those described in individual report #Z01 HL 02312 02 CHB) represent the first attempts to treat genetic disease by manipulation of gene expression.

Proposed Course of the Project:

A systematic examination of various drug dosage and interval of administration will be completed to assess the most effective regimen. The peak HbF levels achieved following 2-3 months of treatment will be determined in several patients with sickle cell anemia. If the increase in HbF production is a consistent and reproducable response, consideration will be given to long term evaluation of drug efficacy.

Publications:

None

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL, HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 through September 30, 1984

Atherosclerosis is the underlying basis of most coronary artery disease and coronary artery disease is the leading cause of death in the United States. Because pathological cholesterol accumulation is central to the development of the atherosclerotic lesion, we have chosen to study this aspect of atherosclerosis.

Work in the Laboratory of Experimental Atherosclerosis during the past year has extended our previous investigations of cholesterol deposition within atherosclerotic lesions. Previous work in our laboratory established, using histochemical techniques, that separate free cholesterol-containing and cholesteryl ester-containing particles accumulate within human and experimentally induced atherosclerotic lesions. Last year, we were successful in developing a protocol for isolation of these cholesterol-rich particles from atherosclerotic vessels. This year, we have completed a comprehensive analysis of the lipid and protein contents of these particles isolated from both human and rabbit (normal and atherosclerotic) aortas.

Particles isolated from the aortas of humans and cholesterol-fed rabbits contained free cholesterol, cholesteryl esters, protein, phospholipids, and triglycerides. Interestingly, many of the isolated particle fractions contained cholesterol that was predominantly unesterified confirming our previous histochemical observations.

Negative staining of the lipid particles showed them to be of similar sizes in control and experimental groups (120 angstroms). The major protein found in all the isolated particles was of about 66,000 M.W. as determined by SDS polyacrylamide gel electrophoresis. No proteins corresponding to apo-A or apo-B were observed. The lipid and protein composition of the particles indicates that these novel aortic lipoproteins do not resemble serum lipoproteins.

Particles isolated from aortas of control rabbits contained protein, phospholipids, and triglycerides similar to particles isolated from aortas of humans and cholesterol-fed rabbits. However, no detectable free cholesterol or cholesteryl ester occurred in the particles isolated from aortas of control rabbits. To our knowledge, this is also the first report of the presence of these particles. The presence of these lipoprotein particles in the control aorta suggests that they may participate in the normal regulation and control of lipid transport in the aorta. Their similarity in protein composition to that of the lipoprotein particles found in the aortas of humans and cholesterol-fed rabbits suggests that they may accumulate cholesterol in the atherosclerotic aorta. Future studies will be carried out to determine the origins and interrelationships of these novel aortic lipoproteins.

We have made progress this year in defining a mechanism that may explain pathological accumulation of cholesterol within atherosclerotic lesions. Because our earlier histochemical studies indicated that cholesterol-containing particles accumulated not only within the vessel wall but also within thrombi

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associated within vascular lesions, we examined the role of thrombosis in cholesterol deposition.

Last year we demonstrated that cholesterol-containing particles (similar to those observed within atherosclerotic lesions) appeared in vitro within plasma that was clotted, but only when platelets were present. Smooth muscle cells, when added to the platelet-containing clots, accumulated these cholesterol-containing particles. No cholesterol accumulated in smooth muscle cells added to plasma clots lacking platelets. Thus, platelets were able to mediate cholesterol accumulation within smooth muscle cells.

This year we have examined platelet-mediated cholesterol accumulation by smooth muscle cells under more defined conditions in tissue culture. We have found that when washed platelets were incubated with cultured smooth muscle cells in the presence of a platelet-activating agent such as thrombin, cholesteryl ester lipid droplets accumulated within the smooth muscle cells. This occurred in the absence of serum or serum lipoproteins. Cholesteryl ester accumulation was not dependent on de novo cholesterol synthesis and occurred with platelet-free supernatants prepared from activated platelets. These findings suggest that thrombin-stimulated (i.e., activated) platelets release cholesterol in some form that can be taken up and stored in the cultured smooth muscle cells. Preliminary chemical analysis of supernatants prepared from the activated platelets indicates that platelets do release cholesterol in response to thrombin. Studies will be carried out to isolate and characterize the nature of cholesterol released by activated platelets.

The findings of this study suggest that platelet-mediated cholesteryl ester lipid droplet accumulation in vascular-derived smooth muscle cells may be a mechanism that could explain the cholesterol accumulation that occurs within atherosclerotic lesions. Platelets have been previously implicated in the atherosclerotic process. Induction of vessel-associated thrombi in experimental animals is associated with the development of lipid-containing atherosclerotic lesions. This occurs even in the absence of elevated serum cholesterol levels. Lesion development is inhibited in these animals when they are made thrombocytopenic.

The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions has been previously recognized. Our research has shown that platelets can mediate cholesterol ester lipid droplet accumulation within cultured vascular-derived smooth muscle cells. The possible role of platelets in mediating cholesterol accumulation within atherosclerotic lesions must now also be considered in evaluating the pathogenesis of atherosclerosis.

Work has continued this year concerning the quantification of foam cells (i.e., cholesterol ester-containing cells) using flow cytometry in spontaneous and experimentally induced atherosclerosis in swine. For the study of foam cells in spontaneously occurring atherosclerosis, male and female animals (ages 2 to 12 years) that had been maintained on a low-fat, cholesterol-free diet throughout their lifetimes were utilized. For the study of the appearance of foam cells during experimentally induced atherosclerosis, a second group of animals, all males under one year of age, were begun on a high-fat (supplied as lard), high-cholesterol diet. After 3 months, a subset of these cholesterol-fed

animals was switched to a normal, low-fat, cholesterol-free diet to study the incidence of foam cells during regression of atherosclerosis. Appropriate control animals were maintained on a low-fat, cholesterol-free diet throughout the experimental period. Vascular tissues obtained from the swine were processed and evaluated using flow cytometric analysis as described in project report Z01 HL 02824-05 EA.

In cholesterol-lard-fed swine, serum cholesterol levels increased to a maximum of 4 times the cholesterol levels of animals fed the control diet. Foam cell number increased on the cholesterol-lard diet up to 3 months. Between 3 and 5 months, there was a decrease in foam cell number. This decrease (possibly indicating foam cell necrosis), may not be statistically significant because of the large variation in foam cell numbers observed for animals within similar treatment groups. This variation appears to be correlated with the variation in serum cholesterol levels experienced by different animals. After 5 months, foam cell numbers again increased. Even though serum cholesterol increased in lard-fed animals (to approximately 1.5 times the serum cholesterol in control-fed animals), foam cell numbers did not increase.

When animals were fed the cholesterol-lard diet for 3 months and then switched to the control diet, their elevated serum cholesterol levels decreased to normal within 1 month and they experienced no further increase in foam cell number. Foam cell numbers per mm^2 of aorta increased similarly in the thoracic and abdominal aortic regions. Foam cell numbers per mm^2 of aorta for each group at 6 months was as follows: control-fed, 30; lard-fed, 30; cholesterol-lard-fed, 5320; cholesterol-lard-fed (3 mo) followed by control-fed (3 mo), 1700.

Our results also indicate that aged animals maintained on cholesterol-free diets their entire lifetimes do have foam cells within their aortas. The finding that animals never fed cholesterol, nevertheless develop foam cells is highly significant. Also significant is the finding that feeding of cholesterol but not feeding of lard (i.e., saturated fat) accelerates the development of foam cells. This accelerated development can be reversed when cholesterol is removed from the diet.

Flow cytometry and sorting is a methodology that provides a unique approach to the study of atherosclerosis. The focal nature of atherosclerosis and the low numbers of foam cells occurring in spontaneous or early experimentally induced lesions precludes determinations of foam cell number by other means. Our results indicate that it is possible to quantify and purify foam cells using flow cytometry. Work is proceeding to complete data analysis and correlation of foam cell numbers with serum cholesterol levels, serum lipoprotein-cholesterol levels, age, and a number of other variables.

In conclusion, significant process has been made in the LEA towards our goal of defining the nature of accumulated cholesterol and the mechanism of cholesterol accumulation within atherosclerotic lesions. We have defined novel vessel-associated lipoproteins that may play an important role in the regulation of arterial cholesterol metabolism. We also have shown how platelets may play an important role in causing cholesterol to deposit within vascular cells. In addition, the LEA has provided research support for vascular-related studies being carried out in other NHLBI laboratories (Molecular Disease, Cardiology, and Cardiac Surgery).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02824-05 EA

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Flow cytometric analysis of cells isolated from atherosclerotic lesions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Cupp	Staff Fellow	NHLBI, LEA
Others:	H. S. Kruth	Senior Investigator	NHLBI, LEA

COOPERATING UNITS *(if any)*

Perinatal Toxicology, Division of Toxicology, FDA (M.A. Khan and G.R. Henderson) Lipid Nutrition Lab, USDA (E. Berlin), Laboratory of Statistical and Mathematical Methodology, Division of Computer Research and Technology, NIH (G. Campbell)

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2	1	1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Flow cytometry and sorting is a methodology which provides a unique approach to the study of atherosclerosis. The focal nature of atherosclerosis and the low numbers of foam cells (i.e., cholesteryl ester-rich cells) occurring in spontaneous or early experimentally induced lesions precludes determinations of foam cell numbers by other means. This research has demonstrated that foam cell number can be quantified using flow cytometry.

Six-month-old male swine were maintained for 6 months on control, lard, or cholesterol-lard diets. Some animals were fed the cholesterol-lard diet for 3 months and then fed the control diet for an additional 3 months. Other animals studied were comprised of male and female swine up to 12 years of age that had been maintained on a cholesterol-free diet their entire lifetimes.

Intimal-medial aortic tissue preparations were enzymatically digested using collagenase and elastase. The resulting single-cell suspensions were fixed in formalin. A technique for fluorescent staining of the cholesteryl ester-containing foam cells in these cell preparations was developed. The fluorescence of each of 100,000 stained cells was measured using a flow cytometer. Flow cytometric analysis and sorting of these filipin-stained cell preparations showed that foam cells could be quantified and purified.

Animals never fed cholesterol had foam cells. Foam cells increased in number in cholesterol-lard-fed animals but did not increase in lard-fed or control-fed animals. Foam cells did not continue to increase after cholesterol-lard-fed animals were switched to control diets.

The finding that animals never fed cholesterol, nevertheless develop foam cells is highly significant. Also significant is the finding that feeding of cholesterol but not feeding of lard (i.e., saturated fat) accelerates the development of foam cells. This accelerated development can be reversed when cholesterol is removed from the diet.

Project Description:

Objectives: The objectives of this work were to develop a means of detecting and quantifying foam cells (i.e., cholesteryl ester-containing cells) in atherosclerotic vessels using flow cytometry and sorting. Additional objectives were the following: 1. determination of the incidence of foam cells in spontaneously occurring atherosclerosis in male and female swine of varying ages. 2. determination of the kinetics of the appearance of foam cells during the induction of atherosclerosis by cholesterol-lard feeding. 3. determination of the incidence of foam cells during regression of atherosclerosis induced by switching cholesterol-lard-fed animals to normal, control diets. 4. the correlation of foam cell incidence with total serum cholesterol and lipoprotein-cholestrol levels.

Methods: Swine were used to provide the vascular tissues for these studies. For the study of foam cells in spontaneously occuring atherosclerosis, male and female animals (ages 2 to 12 years) that had been maintained on a low-fat, cholesterol-free diet throughout their lifetimes, were utilized. For the study of the appearance of foam cells during induced atherosclerosis, a second group of animals, all males under one year of age, were begun on a high-fat (supplied as lard), high-cholesterol diet. After 3 months, a subset of these cholesterol-fed animals was switched to a normal, low-fat, cholesterol-free diet to study the incidence of foam cells during regression of atherosclerosis. Appropriate control animals were maintained on a low-fat, cholesterol-free diets throughout the experimental period. Vascular tissues obtained from the swine were processed and evaluated using flow cytometric analysis.

Single cell preparations for flow cytometric analysis were prepared in the following way. The intimal and part of the medial layer of vascular cells was stripped from the aorta using a surgical dermatome. Prior to stripping, the aortic area was traced onto aluminum foil and later measured using planimetry. The aortic tissue was enzymatically dissociated into a single-cell suspension using collagenase and elastase. Then, cells were fixed in formalin and stored until staining. The total amount of DNA was measured for each cell sample to determine the percent recovery of cells from the original tissue. This allowed computation of the total number of foam cells present in each vessel or the number of foam cells per unit of vessel surface area.

The following staining procedure was developed to stain foam cells. First, unesterified cholesterol was removed from cells by ethanol extraction. Then, cells were treated with cholesterol esterase to convert cellular esterified cholesterol to unesterified cholesterol. The released unesterified cholesterol was then specifically stained with the fluorescent dye, filipin. Control cells were processed in a similar way except that either enzyme treatment or filipin staining was omitted. After mild sonication to disaggregate any cell clusters that may have formed, the foam cells were counted and/or sorted using the fluorescence-activated cell sorter.

Major Findings: Our results indicate that it is possible to quantify and purify foam cells using flow cytometry. In cholesterol-lard-fed swine, serum cholesterol levels increased to a maximum of 4 times the cholesterol levels of animals fed the control diet. Foam cell number increased on the

cholesterol-lard diet up to 3 months. Between 3 and 5 months, there was a decrease in foam cell number. This decrease (possibly indicating foam cell necrosis) may not be statistically significant because of the large variation in foam cell numbers observed for animals within similar treatment groups. This variation appears to be correlated with the variation in serum cholesterol levels experienced by different animals. After 5 months, foam cell numbers again increased. Even though serum cholesterol increased in lard-fed animals (to approximately 1.5 times the serum cholesterol in control-fed animals), foam cell numbers did not increase.

When animals were fed the cholesterol-lard diet for 3 months and then switched to the control diet, their elevated serum cholesterol levels decreased to normal within 1 month and they experienced no further increase in foam cell number. Foam cell numbers per mm^2 of aorta increased similarly in the thoracic and abdominal aortic regions. Foam cell numbers per mm^2 of aorta for each group at six months was as follows: control-fed, 30; lard-fed, 30; cholesterol-lard-fed, 5320; cholesterol-lard-fed (3 mo) followed by control-fed (3 mo), 1700. Our results also indicate that aged animals maintained on cholesterol-free diets their entire lifetimes do have foam cells within their aortas. Analysis of these data are not yet complete.

Significance to Biomedical Research and the Program of the Institute: Flow cytometry and sorting is a methodology that provides a unique approach to the study of atherosclerosis. The focal nature of atherosclerosis and the low numbers of foam cells occurring in spontaneous or early experimentally induced lesions precludes determinations of foam cell numbers by other means. This research has demonstrated that foam cell number can be quantified using flow cytometry.

The finding that animals never fed cholesterol, nevertheless develop foam cells is highly significant. Also significant is the finding that feeding of cholesterol but not feeding of lard (i.e., saturated fat) accelerates the development of foam cells. This accelerated development can be reversed when cholesterol is removed from the diet.

Proposed Course: Continued analysis and correlation of foam cell numbers, serum cholesterol levels, serum lipoprotein-cholesterol levels, age, and other variables will be carried out.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02826-03 EA

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and characterization of lipid-rich particles in atherosclerotic lesions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. Gamble	Guest Worker	NHLBI, LEA
Others:	H. S. Kruth	Senior Investigator	NHLBI, LEA

COOPERATING UNITS *(if any)*

Lab of Cellular and Developmental Biology, NIADDK (M. Sliwkowski), Section on Lab Animal Medicine and Surgery, NHLBI (J. Pierce)

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A protocol was developed that permitted the isolation of distinct populations of free cholesterol and cholesteryl ester-containing particles. The particles were first observed in atherosclerotic lesions in this laboratory using histochemical techniques.

The lipid composition of these particles was determined. Particles isolated from the aortas of humans and cholesterol-fed rabbits contained free cholesterol, cholesteryl esters, protein, phospholipids, and triglycerides. The predominantly cholesteryl ester-containing particles had densities of 1.000-1.002 and the predominantly free cholesterol-containing particles had densities of 1.002-1.098. Particles isolated from aortas of control rabbits contained protein, phospholipids, and triglycerides similar to particles isolated from aortas of cholesterol-fed rabbits. However, no detectable free cholesterol or cholesteryl ester occurred in particles isolated from control aortas.

The major protein found in all the particles was of about 66,000 M.W. as determined by SDS polyacrylamide gel electrophoresis. No proteins corresponding to apo-A or apo-B were observed. Negative staining of the particles showed them to be of similar sizes in control and experimental groups (120 angstroms). The lipid composition of the particles and their major protein of 66,000 M.W. indicate that these novel aortic lipoproteins do not resemble serum lipoproteins.

A population of particles in control rabbit aortas containing no detectable free cholesterol or cholesteryl ester has been identified. To our knowledge, this is also the first report of the presence of these particles. The presence of such particles in control aortas suggests that they may participate in the normal regulation and control of lipid transport in the aorta. Their similarity in protein composition to that of the particles found in the aortas of humans and cholesterol-fed rabbits suggests that they may participate in the accumulation of lipid in the atherosclerotic aorta.

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Project Description

Objective: The purpose of this project was to develop a protocol for the isolation and analysis of the free cholesterol and cholesteryl ester-containing particles previously identified in atherosclerotic lesions by histochemical methods in this laboratory. The analysis included the determination of phospholipids, triglycerides, proteins, cholesterol, and cholesteryl esters in the isolated particles.

Methods: Aortas from New Zealand white male rabbits, maintained on cholesterol or control diets, were dissected to obtain intima-media preparations. Intima-media preparations of human aortas were similarly prepared. The aortas were homogenized with a Tekmar tissue homogenizer in 0.1M tris-HCl buffer (pH 7.4) containing 0.02% (w/v) sodium azide. Homogenates were centrifuged at 264 x g for ten minutes and 1995 x g for five minutes to obtain a tissue-free supernatant. The supernatant was filtered in a Nuclepore stirred cell through a 5μ polycarbonate filter. The filtrate was concentrated on a YM10 Amicon filter. The concentrated supernatant was centrifuged at 170,000 x g in a SW41 Beckman rotor in 26.5% sucrose (density 1.1 g/ml) at 4°C for 15 hours. The fraction which floated to the top of the centrifuge tube was collected and recentrifuged in a 0 - 26.5% continuous sucrose gradient in an SW41 Beckman rotor for 21 hours at 170,000 x g. Fractions (0.6 ml) of the gradients were collected using a Buchler Densi-flow II gradient collector system. Fractions were pooled to define the visible and interband areas observed along the gradient.

Each pooled fraction was analyzed for protein, phospholipid, cholesterol, cholesteryl ester, and triglyceride content. Quantitative determination of protein was by the method of Lowry. Phospholipid was measured spectrophotometrically by the method of Chalvardjian and Rudnicki. Cholesterol and cholesteryl ester were determined fluorometrically by the method of Gamble et al. Triglyceride was measured fluorometrically by the method of Chernick. The sizes of the isolated particles was determined using negative staining and electron microscopic analysis. Qualitative identification of the proteins was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Major Findings: A protocol was developed that permitted the isolation of lipid particles having either free cholesterol or cholesteryl ester as the predominant cholesterol form. The predominantly cholesteryl ester containing-particles had densities of 1.000-1.002. The predominantly free cholesterol-containing particles had densities of 1.002-1.098. The compositions of the particles are shown in the tables on the pages following.

Electrophoretic analysis of particle proteins showed the presence of a major band of about 66,000 M.W. in each of the pooled fractions. No bands corresponding to apo A and apo B were observed. Negative staining and electron microscopic analysis showed the particles to be 120 Å in size. In concentrated solution, the particles aggregated yielding apparently large polymeric aggregates. The control aorta contained particles of similar size and density ranges but did not contain detectable cholesterol or cholesteryl ester.

Composition of Particles Isolated From Rabbit Aortas (weight percent)

A) Cholesterol-Fed

	Density g/ml	Protein	Phospho-lipid	Cholest. Total	Cholest. Free	Cholest. Ester	Trigly-ceride
I.	1.000-1.002	13.2	34.7	39.2	6.9	32.2	12.7
II.	1.002-1.006	8.2	52.3	39.4	25.6	13.7	0
III.	1.006-1.030	18.2	77.1	4.6	4.4	0.2	0
IV.	1.030-1.067	14.0	61.8	15.2	13.1	2.1	8.9
V.	1.067-1.091	21.3	41.0	15.7	10.7	4.9	21.8
VI.	1.091-1.098	8.0	16.5	2.5	2.3	0.1	72.9

B) Control-Fed

I.	1.000-1.002	11.4	12.6	0.0	0.0	0.0	75.9
II.	1.002-1.006	4.2	11.7	0.0	0.0	0.0	84.0
III.	1.006-1.030	14.4	10.1	0.0	0.0	0.0	75.4
IV.	1.030-1.067	12.2	7.0	0.0	0.0	0.0	80.7
V.	1.067-1.091	5.5	6.2	0.0	0.0	0.0	88.2
VI.	1.091-1.098	8.3	8.4	0.0	0.0	0.0	83.2

Composition of Particles Isolated From Human Aortas (weight percent)

I.	1.001	39.3	41.6	15.6	2.8	12.8	3.1
II.	1.001	39.7	32.7	7.7	2.6	5.2	19.8
III.	1.011	33.2	37.6	22.2	15.4	6.8	6.9
IV.	1.027	25.9	31.5	18.1	9.0	9.1	24.5
V.	1.047	37.4	24.0	12.2	5.0	7.2	26.3
VI.	1.058	39.9	23.7	2.6	2.1	0.5	33.8
VII.	1.074	73.6	17.1	2.2	1.4	0.8	7.0

Composition of Particles Isolated From Rabbit Aortas (μgrams per aorta)

A) Cholesterol-Fed

	Density g/ml	Protein	Phospho-lipid	Cholest. Total	Cholest. Free	Cholest. Ester	Trigly-ceride
I.	1.000-1.002	20.9	54.7	61.7	10.9	49.3	20.1
II.	1.002-1.006	116.1	738.4	555.8	361.6	194.2	0
III.	1.006-1.030	303.7	1285.8	77.6	73.5	4.1	0
IV.	1.030-1.067	109.2	481.2	118.2	101.8	16.4	69.8
V.	1.067-1.091	82.9	159.2	61.0	41.7	19.1	84.8
VI.	1.091-1.098	33.2	66.2	10.1	9.4	0.6	292.3

B) Control-Fed

I.	1.000-1.002	4.2	4.6	0	0	0	27.8
II.	1.002-1.006	13.8	5.0	0	0	0	36.0
III.	1.006-1.030	6.6	4.6	0	0	0	35.3
IV.	1.030-1.067	7.5	4.2	0	0	0	49.2
V.	1.067-1.091	4.6	5.1	0	0	0	48.6
VI.	1.091-1.098	4.9	5.1	0	0	0	48.8

Significance to Biomedical Research and the Program of the Institute: A protocol was developed that permitted the isolation of distinct populations of free cholesterol and cholesteryl ester-containing particles previously observed histochemically in atherosclerotic lesions. The lipid composition of the particles and their major protein of 66,000 M.W. indicate that these novel aortic lipoproteins do not resemble serum lipoproteins.

A population of particles in control rabbit aortas containing no detectable free cholesterol or cholesteryl ester has been identified. To our knowledge, this is the first report of the presence of these particles. The presence of such particles in the control aorta suggests that they may participate in the normal regulation and control of lipid transport in the aorta. Their similarity in protein composition to that of the particles found in the aorta of cholesterol-fed rabbits suggests that they may participate in the accumulation of lipid in the atherosclerotic aorta.

Proposed Course: Studies will be carried out to determine the origins and interrelationships of the novel aortic lipoproteins described above.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02827-02 EA

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Platelet-mediated cholesterol accumulation within vascular-associated cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H. S. Kruth Senior Investigator NHLBI, LEA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pathological deposition of cholesterol underlies the development of vascular atherosclerotic lesions. The mechanism of cholesterol deposition is not known. This research project shows that platelets can mediate cholesterol accumulation within aortic smooth muscle cells.

Washed platelets prepared from rat blood were incubated with cultured rat aortic smooth muscle cells in the presence or absence of thrombin. All experiments were carried out in the absence of serum. Cholesterol accumulation within smooth muscle cells was detected histochemically using filipin staining.

Cholesteryl ester lipid droplets accumulated in smooth muscle cells only when added platelets were activated by thrombin. Addition of increasing numbers of platelets resulted in increasing amounts of cholesteryl ester within cells. Cholesteryl ester accumulation occurred in confluent and non-confluent smooth muscle cell cultures. Addition of the cholesterol synthesis inhibitor mevinolin to smooth muscle cell cultures incubated with thrombin-activated platelets, did not diminish cholesteryl ester lipid droplet accumulation.

Platelet-free supernatants were prepared from thrombin-activated platelets incubated separately from smooth muscle cells. When added to the cultured smooth muscle cells, the platelet-free supernatants also induced cholesteryl ester lipid droplet accumulation.

These and other results indicate that activated-platelets secrete cholesterol that vascular-derived smooth muscle cells can accumulate. The significance of this research lies in the identification of a mechanism that can explain the pathologic accumulation of cholesterol in smooth muscle cells within atherosclerotic lesions.

Y6 P

Project Description:

Objectives: In previous work (see reports Z01 HL 02827-01 and Z01 HL 02826-02 of last year) we identified unusual lipid particles within human and experimentally induced atherosclerotic lesions. These particles contain predominantly unesterified cholesterol as opposed to esterified cholesterol and had not been previously described. Last year, we demonstrated that similar particles could be generated in vitro within plasma clots but only when platelets were added to the clots. Smooth muscle cells that were added to the platelet-containing clots accumulated the cholesterol-rich particles. This year we have examined this platelet-mediated cholesterol accumulation by smooth muscle cells under more defined conditions in tissue culture.

Methods: Platelet-rich plasma has been prepared from venous blood of rats. Platelets were isolated from the platelet-rich plasma by centrifugation and washed repeatedly in a phosphate-buffered saline solution. For experiments, cultured aortic smooth muscle cells were maintained at least three days in the absence of serum. This procedure eliminated any stored lipid that may have accumulated in cells during previous growth in serum-containing culture medium. All experiments were carried out in the absence of serum and in the absence of serum lipoproteins.

Varying amounts of washed platelets were added to the cultured smooth muscle cells in the absence or presence of thrombin to activate the platelets. In some experiments, washed platelets were incubated in the absence of smooth muscle cells. Platelet-free (filtered or centrifuged) supernatants from these incubations were then added to smooth muscle cell cultures. Cultures were incubated overnight and fixed in formalin. Accumulated cholesteryl ester was stained with oil red O or was hydrolyzed enzymatically so that released unesterified cholesterol could be specifically detected with the fluorescent stain filipin. Smooth muscle cells were also examined using the electron microscope.

Major Findings: Smooth muscle cells accumulated numerous cholesteryl ester lipid inclusions when incubated with thrombin-activated platelets. These cells contained oil red O-stained lipid droplets similar to the oil red O-stained lipid droplets that accumulate in smooth muscle cells within atherosclerotic lesions. Electron microscopic examination of the cultured smooth muscle cells revealed that non-membrane-bound lipid droplets had accumulated. No lipid inclusions accumulated in smooth muscle cells of cultures incubated with unactivated platelets (i.e., in absence of thrombin). Thrombin, in the absence of platelets, had no effect on lipid droplet accumulation by smooth muscle cells. Addition of increasing numbers of platelets (7.5×10^8, 15×10^8, and 30×10^8 per culture) resulted in increasing amounts of cholesteryl ester within cells. Cholesterol accumulation occurred in both non-confluent and confluent smooth muscle cell cultures.

To determine whether thrombin-stimulated platelets released a factor that induced cholesteryl ester lipid droplet accumulation, 30×10^8 washed platelets were incubated in single well slide chambers with and without thrombin for 24 hours in the absence of smooth muscle cells. Supernatants from these

cultures were harvested and centrifuged at 10,000 g for 10 min or were centrifuged at 1000 g for 3 min and filtered (0.4 μ) to remove all platelets. Both supernatants caused cholesteryl ester lipid droplet accumulation when added to and incubated 24 hours with smooth muscle cells. Supernatants from platelets incubated in the absence of thrombin and then added to and incubated with smooth muscle cell cultures did not promote cholesteryl ester lipid droplet accumulation. Supernatants from platelets incubated varying lengths of time (30 min, 1, 3, 24, or 48 hours) before addition to and incubation with smooth muscle cell cultures for 24 hours, resulted in increasing amounts of cholesteryl ester lipid accumulation. However, supernatants from thrombin-activated platelets incubated 48 hours before addition to smooth muscle cell cultures did not promote greater cholesteryl ester accumulation than supernatants from thrombin-activated platelets incubated 24 hours.

Platelet-mediated accumulation of cholesteryl ester lipid droplets in smooth muscle cells did not depend on de novo cholesterol synthesis. Addition of the cholesterol synthesis inhibitor mevinolin to smooth muscle cell cultures incubated with thrombin-activated platelets, did not diminish cholesteryl ester lipid droplet accumulation.

The fact that platelet-free supernatants could induce cholesteryl ester accumulation indicates that cholesteryl ester accumulation did not simply result from endocytosis of activated platelets by the smooth muscle cells. Thus, it is likely that thrombin-activated platelets release cholesterol in some form that can be taken up by smooth muscle cells and stored as cholesteryl ester lipid droplets. Preliminary chemical analysis of activated platelet supernatants indicate that this is the case.

Significance to Biomedical Research and the Program of the Institute: The findings of this study suggest that platelet-mediated cholesterol ester lipid droplet accumulation in vascular-derived smooth muscle cells may be the mechanism of cholesterol accumulation that occurs within atherosclerotic lesions. Platelets have been previously implicated in the atherosclerotic process. Induction of vessel-associated thrombi in experimental animals is associated with the development of lipid-containing atherosclerotic lesions. This occurs even in the absence of elevated serum cholesterol levels. Lesion development is inhibited in these animals when they are made thrombocytopenic.

The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions has been previously recognized. Our research has shown that platelets can mediate cholesterol ester lipid droplet accumulation within cultured vascular-derived smooth muscle cells. The possible role of platelets in mediating cholesterol accumulation within atherosclerotic lesions must now also be considered in evaluating the pathogenesis of atherosclerosis.

Atherosclerosis is the underlying basis of most coronary artery disease and coronary artery disease is the leading cause of death in the United States. Because pathological cellular cholesterol accumulation is central to the atherosclerotic process, our new findings concerning the role of platelets in mediating cellular cholesterol accumulation are of great significance to the program of the NHLBI.

Proposed Course: Studies will be carried out to isolate and characterize the nature of cholesterol released by activated platelets. It will be determined whether drugs can be used to inhibit platelet release of cholesterol.

Publications:

1. Kruth, H.S.: Filipin-positive particles, oil red O-negative particles in atherosclerotic lesions induced by cholesterol feeding. Lab. Invest. 50:87-93, 1983.
2. Kruth, H.S.: Localization of unesterified cholesterol in human atherosclerotic lesions. Demonstration of filipin-positive, oil red O-negative particles. Amer. J. Pathol. 114:201-208, 1984.
3. Kruth, H.S.: Histochemical detection of esterified cholesterol within human atherosclerotic lesions using the fluorescent probe filipin. Atherosclerosis, 51:281-292, 1984.
4. Kruth, H.S. and Fry, D.L.: Histochemical detection and differentiation of free and esterified cholesterol in swine atherosclerosis using filipin. Exp. Molec. Pathol. 40:288-294, 1984.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 through September 30, 1984
Section of Experimental Therapeutics

In this year, the Experimental Therapeutics Section of the Hypertension-Endocrine Branch has continued its basic and clinical research into many aspects of the causes and therapy of hypertension. These studies have focused on the neurohumoral and vasoactive systems that control the circulation.

I. Catecholamines and the sympathetic nervous system. We have been able to assess the contribution of sympathetic outflow to blood pressure in patients with essential hypertension by measuring blood pressure and plasma norepinephrine responses to clonidine, an antihypertensive drug which decreases central sympathetic outflow. Clonidine decreased mean arterial pressure and plasma norepinephrine in all patients and controls. Among the hypertensives, resting plasma norepinephrine was significantly related to the decrease in mean arterial pressure after clonidine ($r=0.62$, $p<0.001$). The magnitude of the depressor response in the patients also was correlated significantly with the decrease in plasma norepinephrine after clonidine ($r=0.60$, $p<0.001$). In contrast, no relationship was obtained between the magnitude of the depressor response and either baseline norepinephrine or the decrease in norepinephrine after clonidine among normotensive subjects. These results suggest that increased sympathetic outflow plays a pathophysiologic role in some patients with essential hypertension. We were surprised to find no relationship between the fall in pressure and the fall in plasma norepinephrine after clonidine among the normotensives, since our findings and those of several other groups indicated a clear-cut relationship among the hypertensives. These results suggest that clonidine may have a second mechanism of antihypertensive action besides inhibition of central sympathetic outflow. The combination of a high resting level of plasma norepinephrine and a large fall in pressure after clonidine serves to identify patients with essential hypertension in whom increased sympathetic outflow contributes to their high blood pressure. We have previously shown that basal levels of plasma norepinephrine are significantly elevated in a significant number of patients with essential hypertension. Now we have measured norepinephrine and isoproterenol kinetics in patients with essential hypertension as well as in normotensive control subjects in order to determine if abnormalities in the disappearance of these substances from circulating plasma indicate malfunctions of the sympathetic nervous system or neuronal norepinephrine uptake. We infused tracer labeled l-norepinephrine, d-norepinephrine, and dl isoproterenol. We found that: a) increased plasma norepinephrine levels seen in some patients with essential hypertension result from increased sympathetic neural activity and not from decreased clearance of norepinephrine, b) changes in the isoproterenol to norepinephrine ratio after the simultaneous infusion of both provide an index of neuronal norepinephrine uptake in man, and c) neuronal norepinephrine removal is not stereospecific. Patients with idiopathic orthostatic hypotension had significantly lower baseline plasma norepinephrine levels than either normotensive controls or patients with multiple system atrophy. This decreased average level of plasma norepinephrine was due to decreased spillover, not accelerated norepinephrine

clearance. Our data also suggested that patients with idiopathic orthostatic hypotension have decreased sympathetically mediated norepinephrine release from neuron terminals at rest and have deficient neuronal norepinephrine removal. When disappearance kinetics of isoproterenol and norepinephrine were compared in brachial arterial and antecubital venous plasma, we found that: 1) the arm removes about half of the norepinephrine reaching it, 2) the arm thus contributes importantly to the norepinephrine concentration in antecubital blood, and 3) this neuronal uptake was abolished in subjects pretreated with desipramine, a blocker of neuronal norepinephrine uptake.

We have continued to improve our technology for the measurement of plasma catecholamines via liquid chromatography, coupled with electrochemical detection. We can now readily measure DOPA and dopamine as well as epinephrine and norepinephrine. We have noted that approximately 33% of the plasmas we have assayed for catecholamines have shown an additional peak that is larger than that of norepinephrine, epinephrine, or dopamine with a retention time between that of epinephrine and dopamine. We have found that this peak was due to dihydrocaffeic acid, a metabolite of caffeic acid that is a major constituent of food substances made from either coffee or cola beans. Thus our further studies will proscribe drinking of coffee or cola in any form for at least 18 hours prior to blood sampling. When we reassessed the relationship between venous plasma levels of norepinephrine and subject age in patients with primary or secondary hypertension and in normotensive controls, we found that plasma norepinephrine increased significantly with subject age in the normotensive subjects, but that there was no age related increase among hypertensives. When subjects less than 40 years old were considered, hypertensives had significantly higher plasma norepinephrine than controls, but above the age of 40 years, no significant hypertensive-normotensive difference was obtained. Among patients with secondary hypertension of various causes, only the group with renal parenchymal disease had elevated levels of supine plasma norepinephrine. These results suggest that increased sympathetic neural activity occurs in the early stages of essential hypertension, and that there is no apparent abnormality of plasma norepinephrine in secondary forms of clinical hypertension, with the exception of bilateral renal parenchymal disease, where defective norepinephrine clearance may be present.

In previous studies we have shown that there is an inverse relationship between baroreflex cardiac sensitivity and plasma norepinephrine in patients with essential hypertension. This finding could have resulted from excessive stress responses to the experimental situation in some patients with hypertension. If this were true, then the relationship should be abolished in sedated subjects. Therefore we have repeated the assessment of baroreflex sensitivity in patients after sedation with intravenous diazepam. Preliminary results suggest that sedation does not affect baroreflex sensitivity, but does decrease levels of plasma norepinephrine. This would suggest that high levels of plasma norepinephrine in some hypertensives result from excessive stress responses, but that sedation does not normalize baroreflex sensitivity.

Malignant pheochromocytoma is a very rare tumor. There are no guidelines for chemotherapy of this disease. Recently we were confronted by several young men who had rapidly progressing malignant pheochromocytoma with life-threatening distant metastases and poorly controlled hypertension, in spite of therapy with large doses of both antiadrenergic drugs and an inhibitor of catecholamine

synthesis. We noted that these tumors resembled malignant neuroblastomas, in that they both arise from the same embryonic neural crest tissue and both produce catecholamines. Since neuroblastomas are much more common tumors, systematic studies have yielded good data on the efficacy of various chemotherapeutic regimens. Therefore, we decided to treat these patients with a regimen of cyclophosphamide, vincristine, and dacarbazine on repeated cycles of 21 days. Each patient had a decrease in blood pressure within the first few cycles of treatment and this correlated with significant reductions in plasma and urinary catecholamines and objective regression of tumor size. Four to eleven months after starting therapy, each patient continues to receive chemotherapy with stable disease in one patient and continuing regression of tumor in the other two. In each patient, the blood pressure is well controlled with minimal or no antihypertensive therapy. There have been few side effects and dose limiting toxicity has been due to mild myelosuppression and neurotoxicity. Each patient has been markedly improved, both in terms of his level of activity and his sense of well being. We are all greatly encouraged by these gratifying results and look forward to treating more patients with malignant pheochromocytoma with this therapeutic regimen.

We recently had the opportunity to measure arterial and venous plasma catecholamines and reflexive skin microcirculatory responses in a patient with a lumbar sympathectomy. In this patient we were able to show for the first time that the arterial venous increment in norepinephrine across a sympathetically enervated bed is directly related to sympathetically mediated norepinephrine release in that bed. We could also show via laser Doppler flowmetry that skin microvascular oscillations in blood flow do not depend on fluctuations in sympathetic tone so much as on myogenic activity.

We have measured plasma levels of norepinephrine and pressor responses during sympathetic stimulation or norepinephrine infusion in pithed, vagotomized, alpha-2 adrenoreceptor-blocked, adrenal-demedullated rats with and without blockade of neuronal norepinephrine uptake using desipramine. We have shown that for an increment of 50 torr in mean arterial pressure, the estimated mean junctional norepinephrine concentration was about 7 nM/liter. Norepinephrine concentration gradients between the site of norepinephrine release from sympathetic nerve endings and its appearance in plasma appeared to be reciprocal and equal for sympathetic stimulation and for norepinephrine infusion because these gradients were reduced equally, by about two-thirds, after desipramine treatment. The results indicate that removal of both released and infused norepinephrine is mainly via neuronal uptake. Similar studies are now underway in patients with essential hypertension.

Recent investigations have suggested that endogenous dopamine may have a physiologic role as a circulating or local hormone as well as a neurotransmitter. We therefore gave normal male volunteers two-hour infusions of dopamine at concentrations of 0, 0.03, 0.3, and 3.0 ug/kg/minute for two hours on separate, nonsuccessive days. Basal dopamine levels were <0.03 ng/ml. Mean plasma dopamine concentrations of approximately 0.7, 4, and 38 ng/ml were achieved at the various dose levels respectively. Prolactin decreased significantly during the lowest dopamine infusion rate. Plasma LH and norepinephrine excretion increased during the mid-dose infusion, and heart rate, plasma norepinephrine, and sodium excretion increased during the high dose infusion. Aldosterone, PRA, TSH, FSH and GH did not change significantly during

dopamine administration. Since peripheral venous dopamine concentrations of 0.5 ng/ml are rarely, if ever, achieved in normal man, and maximal hypophyseal-portal dopamine concentration is probably <10 ng/ml, only PRL and LH are likely to be modulated by circulating dopamine in normal man. Target organ responses observed at higher dopamine concentrations are likely to occur only if such concentrations are found in neural effector junctions or localized tissue sites. To determine more specifically the role of dopamine in the modulation of sodium excretion by the kidney, we studied the natriuretic responses to a 4-hour saline load in normal and hypoadrenal subjects before and after administration of the dopamine antagonist, metoclopramide. There was a significant correlation between the metoclopramide-induced rise in aldosterone and the reduction in sodium excretion, suggesting that the antinatriuretic effect of the dopamine antagonist is mediated by its stimulation of aldosterone secretion from the adrenal glomerulosa. In order to understand the role of intrarenally synthesized dopamine, we administered 3H DOPA into the left renal artery of anesthetized dogs during a water diuresis. Preliminary data indicate that a maximum of 10-15% of urinary dopamine is derived from circulating DOPA, and that >95% of filtered DOPA is either reabsorbed or metabolized to other compounds.

II. Role of calcium in hypertension. We have previously identified two subsets of patients with essential hypertension: those whose blood pressure increased when sodium intake was increased from 9 to 249 mEq/d (salt sensitive), and those whose blood pressure did not change (salt resistant). The salt sensitive patients also showed more sodium retention and weight gain before coming into sodium balance than did the sodium resistant patients. In preliminary studies of calcium and magnesium metabolism, an increase in sodium intake in salt sensitive, hypertensive subjects was associated with an increase in urinary calcium from 8.5 to 19.3 mEq/d, whereas urine magnesium showed little change. These findings suggest that the greater sodium retention in salt sensitive hypertensives may be associated with an increase in calcium excretion and would be compatible with the findings reported by McCarron. These data will be followed up because of the recent great interest in the possible role of calcium deficiency in the production of hypertension. To help us in these studies we have recently established the Quin 2 method for measurement of free intracellular calcium content in our laboratory. Quin 2 is an EGTA derivative which can be loaded into cells to permit measurement of intracellular free ionic calcium based upon spectrofluorometric changes when calcium ions are bound. We have been able to apply this method to lymphocytes, neutrophils, and platelets from man and rat, to thymocytes and splenocytes from rat, and to bovine adrenal medullary cells. In all these cells the normal free intracellular calcium content is approximately 100 nM. This is the same value reported by others using this and other techniques. The level of free ionic calcium in resting lymphocytes is unchanged by incubation in 10^{-3} M. ouabain. However, if calcium is washed out of lymphocytes and then added back in the presence of ouabain, the calcium enters more quickly and rises to a higher final concentration. The use of a potassium free incubation medium causes a further increase in free intracellular calcium content. These results are compatible with the hypothesis that the level of free intracellular calcium is dependent upon the activity of the sodium-potassium ATPase pump. We have found that propranolol added to the medium also causes a faster influx of calcium and a 50% increase in final free intracellular calcium content of lymphocytes. This is opposite to what another group has reported recently in platelets of hypertensive patients who were being treated with propranolol. Thus further work is necessary to explain these

discrepancies and to clarify the usefulness of such measurements of intracellular free calcium in circulating blood cells, and whether or not it bears any relationship to the tone in vascular smooth muscle cells. In preliminary in vitro experiments, we have been able to demonstrate that calcium channel blockade reduces presynaptic norepinephrine release in both the vas deferens and aortic tissue from the rat.

III. The Kallikrein-Kinin system. We have shown previously that kinin peptides release acetylcholine and antagonize the effects of opiates in the longitudinal muscle-myenteric plexus preparation of the guinea pig intestine. We have also shown that aminoglycoside antibiotics block the electrically induced contractions of this preparation. We therefore measured directly via radioimmunoassay the output of kinins by the tissue. We found that the gut of the guinea pig produced kinins and that the output of kinins was inhibited by aminoglycoside antibiotics. Thus we have for the first time good evidence for a role for kinins in modulating the contractions of the intestine. We next studied a series of diverse substances, i.e., trasylol, trisamidines, and amilioride, all kallikrein inhibitors, on the gut and found that instead of inhibiting the electrically induced contractions, they all enhanced the contractions. On the other hand, captopril, which should prolong the action of kinins, inhibited the contractions of the gut. Since the action of all these agents is opposite to what we would expect, further studies are necessary to determine if some other as yet unidentified mechanism is operative.

IV. Peptides. We have shown previously that the dose of beta-endorphin needed to cause a significant stimulation of aldosterone secretion in the dog is within the physiologic range, and that beta endorphin therefore may play a role in the control of aldosterone secretion. Now we have been able to show that synthetic human beta-lipotropin, which is released from the pituitary gland concomitantly with ACTH, produced a significant increase in aldosterone, but not cortisol secretion. While the time course of the increase in aldosterone was similar to that previously described for beta-endorphin, the dose of beta-lipotropin needed to produce an equal potent response was larger. None of the following peptides had any effect on the secretion of either adrenal steroid: synthetic-gamma lipotropin, synthetic beta-melanotropin, the C-terminal portion of gamma-lipotropin, alpha-endorphin, beta-lipotropin, or alpha-endorphin. These data indicate that beta-endorphin is the only beta-lipotropin-derived peptide that selectively stimulates aldosterone production in vivo and that the aldosterone secretory activity is contained in the C-terminal sequence of beta-endorphin.

Leu-enkephalin is an endogenous opioid peptide that can arise from two distinct precursors: proenkephalin and prodynorphin. It had been proposed that the major precursor in the brain was proenkephalin. However, in a series of experiments in the rat, we have been able to show that in the substantia nigra and the posterior pituitary, leu-enkephalin is derived primarily from prodynorphin. This is of considerable interest since it shows that proenkephalin molecules can give rise to a variety of cellular secretory products in different tissues, depending upon the extent and pattern of intracellular processing. Prodynorphin derived peptides are potent kappa-opiate receptor agonists, while leu-enkephalin is a delta-opiate receptor agonist. Thus the same precursor, prodynorphin, may yield ligands for different opiate receptor subtypes that can exert different physiologic effects.

We had shown previously that the hypertensive response to an acute infusion of hypertonic saline in rats is mediated through the action of ADH, since the response is abolished in Brattleboro rats that have a congenital absence of AVP, but is intact in the normal parent strain, Long Evans. However, there was some criticism of this work since it had been done in animals that were anesthetized. We therefore modified our technique so that we could perform repetitive, consistent, and reliable measurements of cardiac output in awake rats via a thermodilution catheter and with a maximum injectate volume of 200 ul of solution at room temperature. We have demonstrated that awake animals will tolerate these manipulations well and show no deterioration in hemodynamics over at least 72 hours. With this new technique we have demonstrated that the increase in blood pressure noted with hypertonic saline in the Long Evans rats is due to a significant increase in total periperhal resistance with a mild decrease in cardiac output. However, in Brattleboro rats there was only a minimal increase in blood pressure accompanied by a 5% increase in cardiac output with no significant change in total peripheral resistance. This is further proof that under certain physiologic conditions AVP may have a potent hypertensive action in awake animals.

V. Sodium flux in cell membranes. Recently there has been a great deal of interest in abnormalities of sodium transport and in intracellular sodium content in various models of hypertension in rats. It has been proposed that some of these abnormalities may be the cause of the hypertension. However, there has been no previous attempt to examine simultaneously the changes in cell sodium homeostasis and the development of hypertension in these animals. We studied the sodium content and sodium efflux rate constants of thymocytes from male SH and matched Wistar Kyoto rats at 4, 6, 8, and 12 weeks of age. At each age, cell sodium was higher in SHRs, while cell potassium was unchanged. In both groups, total and ouabain-sensitive efflux fell with increasing age. However, SHRs had lower values for total, ouabain-insensitive and furosimide-insensitive efflux at each age studied. We could define no significant difference in the activity of either pump 1 (i.e., sodium-potassium ATPase) or in pump 2 (co-transport) between SHR and Wistar Kyoto. The only consistent difference was in the ouabain- and lasix-insensitive efflux which is due to a passive leak. It is unlikely that the differences in rate constants for pump 1 and 2 have anything to do with hypertension, since they decrease with age in both normotensive and hypertensive rats. The role of the difference in passive leak as a cause of hypertension remains unanswered by these experiments. We applied the same techniques to the study of DOCA salt hypertension in rats. This was done to assess the possible presence of a hypothesized circulating sodium pump inhibitor in this and other volume expanded models of hypertension. When thymocytes from DOCA salt hypertensive rats were incubated in buffer, they had higher total and ouabain-insensitive rate constants for sodium efflux than the controls. When the cells were incubated in serum, all components of sodium transport were increased in both hypertensive and control animals. However, the change was greater in DOCA salt rats for total, ouabain-insensitive, furosemide-insensitive, and ouabain-sensitive rate constants. These findings suggest that the major difference in sodium transport activity of thymocytes in these animals is due to the ouabain-insensitive component, and that this is due to small changes in both pump 2 and passive efflux. The surprising finding is that the addition of fresh serum from DOCA hypertensive animals appeared to stimulate sodium pump activity rather than inhibit it. This is opposite to prevailing theories that propose that a circulating factor may

inhibit pump activity. When similar experiments were performed in Dahl salt-sensitive and salt-resistant rats, there were no differences in rate constants for sodium efflux when the animals were on a low-salt diet. However, on a high-salt diet the salt-sensitive rats had a higher intracellular sodium content. When serum was added to the thymocytes, total sodium transport activity increased. This elevation was primarily due to the ouabain and furosemide-insensitive efflux, i.e., passive leak. Further measurement of these changes and of the specific elements of efflux affected are currently underway, but these data fail to demonstrate the presence of an inhibitor of sodium-pump activity in these volume expanded models of hypertension.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01944-03 HE

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Arginine Vasopressin on Blood Pressure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.S. Marks	Guest Worker	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
.4	.4	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued to evaluate the mechanism of the blood pressure response induced by osmotic and nonosmotic stimuli of arginine vasopressin (AVP). To further define the hemodynamics responsible for these changes, a method for the repetitive measurement of cardiac output in awake partially restrained rats was developed. We have proven this technique based on the thermodilution principle to be reliable and reproducible for periods up to 70 hours post catheter placement. The increase in BP noted with hypertonic saline in the Long Evans strain of rats is the result of a significant increase in peripheral resistance with an associated decline in cardiac output. Brattleboro rats had a minimal increase in blood pressure and a 5% increase in cardiac output, with no significant change in peripheral resistance. These findings are consistent with our previously reported results in unrestrained animals. Unrestrained Long Evans and Brattleboro rats increased mean arterial pressure 31 and 5 mm Hg respectively, while in unrestrained rats the changes were 37 and 3 mm Hg respectively.

Project Description: Z01 HL 01944-03 HE

Objectives: We seek to define the role of AVP in the control and maintenance of blood pressure during physiologic manipulations which affect both vascular volume and plasma osmolarity. The rise in blood pressure noted with the administration of hypertonic saline (Am J Physiol 240:H287, 1981) is partially reversed following the administration of a synthetic blocker of AVP. We have chosen to use the Brattleboro strain which has a complete congenital absence of AVP and its intact parent strain, Long Evans, to avoid the problems of incomplete inhibition and partial interaction. Our experimental design will provide the means for defining AVP dependence. In addition, we have developed a method for obtaining systemic hemodynamic measurements repetitively in conscious rats.

Methods: Adult rats are unilaterally nephrectomized one week prior to use. On the day preceding the study the remaining kidney is removed. A catheter is placed in the right external jugular vein and positioned at the level of the right atrium. A catheter is inserted in the left femoral artery and advanced to the aortic bifurcation. The thermodilution microprobe (0.33 mm diameter) is advanced through the left carotid to the level of the aortic valve. Prior to closure, probe placement is verified by thermodilution curve generation. Animals are then placed in speciall designed plexiglass holders which allow free mobility of the forelegs and upper body. Animals are maintained in the air flow hood isolated from outside stimuli, with controlled lighting and temperature. A minimum of 14 hours elapse following surgery before experiments are performed. Arterial pressure, heart rate and core body temperature are monitored continuously. 200 µl of room temperature injectate (NS) are rapidly infused through the venous catheter. Calculation of cardiac output is performed by the Cardiomax II-R computer (Columbus Instruments). Reliability of these data was verified by manual computation from the generated curves. Following each study, catheter and probe placement was visually checked.

Results: The administration of hypertonic saline increased mean arterial pressure in the Long Evans group 43% with an associated 26% decrease in cardiac output and an 81% rise in peripheral resistance. In Brattleboro rats, MAP rose an insignificant 2.8% with a 5% increase in cardiac output, while peripheral resistance was unchanged. This pattern of response is identical to our results in unrestrained animals.

This method for obtaining repetitive, consistent, and reliable cardiac output measurements in awake rats, requires a maximum injectate volume of 200 µl, a temperature differential (rat/injectate of 15°C) and careful probe placement. Our studies have shown that animals tolerate these manipulations well and show no deterioration in hemodynamics over at least 72 hours.

Significance to Biomedical Research and the Program of the Institute: The development of a technique for the measurement of systemic hemodynamics in awake animals can be applied to a large number of experimental situations. Our method does not require blood sampling and can be used repetitively in the same animal. This addition to our previously reported model for the study of renal function in awake animals (Hypertension 4:625-633, 1982) allows us to evaluate simultaneously systemic hemodynamics while obtaining urine collections.

Proposed Course of the Study: We intend to complete the hemodynamic data with hypertonic saline and the use of peritoneal dialysis. To determine the response of the vasculature in the Brattleboro animals to endogenous AVP, cross circulation experiments (LE to Brat) will be performed. The role of Na-K ATPase in the LE response to hypertonic saline will be evaluated through the use of acetylstrophanthidin. In addition, the effect of the hypertonic saline infusion on intracellular sodium concentration and the sodium pump will be evaluated.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01960-02 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Target-organ responses to graded dopamine infusions in man.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.L. Levinson	Senior Staff Fellow	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI
	D.S. Goldstein	Senior Investigator	HE, NHLBI
	J.R. Gill, Jr.	Senior Investigator	HE, NHLBI
	J. Folio	Clin. Nurse Tech.	OD, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent investigations have suggested that endogenous dopamine may have a physiologic role as a circulating or local hormone, as well as a neurotransmitter. The results of this study indicate that prolactin, and possibly LH secretion may be altered by acute changes in hypophyseal-portal vein DA, within the physiologic range. It is unlikely that circulating DA modulates secretion of aldosterone, PRA, or other pituitary hormones in normal subjects. DA concentrations of 4 ng/ml, and possibly much higher, would be needed at local renal sites to affect sodium balance.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives: Until recently, endogenous dopamine was viewed primarily as a precursor for norepinephrine and epinephrine, with minimal physiologic significance outside the central nervous system. Clinical studies now indicate that endogenous dopamine may modulate such processes as pituitary hormone release, aldosterone secretion, and sodium excretion. The purpose of this study was (1) to assess hormonal, renal and neurocirculatory responses to infused dopamine (DA) in healthy volunteers under controlled conditions, and (2) to compare dose-response characteristics over a range of DA concentrations which includes both physiologic and pharmacologic blood levels.

Methods: Healthy male volunteers were placed on a 109 mEq/day sodium intake as Clinical Center in-patients. At one week intervals they received 2-hour infusions of dopamine at concentrations of 0, 0.03, 0.3, and 3.0 µg/kg/min. Blood samples were drawn during each infusion for catecholamines (including dopamine), aldosterone, plasma renin activity (PRA), cortisol, prolactin (PRL), TSH, GH, LH and FSH. Urine was collected at the end of the infusion period for electrolytes and catecholamines.

Results: The infusions in six subjects resulted in mean plasma DA concentrations of approximately 0.7, 4 and 38 ng/ml. Basal DA levels were less than 0.03 ng/ml. PRL decreased significantly during the lowest dopamine infusion rate. Plasma LH and norepinephrine excretion increased during the mid-dose infusion, and heart rate, plasma norepinephrine, and sodium excretion increased during the high-dose infusion. Aldosterone, PRA, TSH, FSH and GH did not change significantly during DA administration.

Significance: Since peripheral venous dopamine concentrations of 0.5 ng/ml are rarely, if ever, achieved in normal man, and maximal hypophysial-portal dopamine concentration is probably less than 10 ng/ml, only PRL and LH are likely to be modulated by circulating dopamine in normal man. Target-organ responses observed at higher dopamine concentrations are likely to occur only if such concentrations are found in neuroeffector junctions or localized tissue sites.

Proposed Course of Study: Termination

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01963-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Clonidine suppression testing in essential hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	P.D. Levinson	Senior Staff Fellow	HE, NHLBI
	R. Zimlichman	Visiting Associate	HE, NHLBI
	R. Stull	Chemist	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have done clonidine suppression tests in patients with essential hypertension and in normotensive control subjects in order to determine if increased sympathetic nervous system activity plays a pathophysiologic role in the high blood pressure of some patients.

GPO 904-917

Project Description:

Purpose of Project: We assessed the contribution of sympathetic outflow to blood pressure in patients with essential hypertension by measuring blood pressure and plasma norepinephrine (NE) responses to clonidine, an anti-hypertensive agent which decreases central sympathetic outflow, in 44 patients and in 41 normotensive control subjects of similar age.

Methods: Blood pressure, pulse rate, and plasma catecholamines were measured in each subject before and 3 hours after a single, oral dose of 300 mcg clonidine. Plasma catecholamines were assayed using liquid chromatography with electrochemical detection using procedures developed and validated in this laboratory (See annual report about assay techniques).

Results and Their Significance: The average baseline values for mean arterial pressure, pulse rate, and antecubital venous plasma NE all were significantly higher in the hypertensive than the normotensive group both before and after clonidine. The distribution of values for plasma NE in the hypertensives was displaced towards higher levels and was wider than the distribution of values for plasma NE in the normotensives, both before and after clonidine. As a result, we did not discern any distinct subgroup of hypertensives with high plasma NE.

Clonidine caused decreased mean arterial pressure and plasma NE in all the patients and controls. Among the hypertensives, resting plasma NE was significantly related to the decrease in mean arterial pressure after clonidine ($r=0.62$, p 0.001). The magnitude of the depressor response in the patients also was correlated significantly with the decrease in plasma NE after clonidine ($r=0.60$, p 0.001). In contrast, no relationship was obtained beween the the magnitude of the depressor response and either baseline NE or the decrease in NE after clonidine among normotensive subjects. The results suggest that increased sympathetic outflow plays a pathophysiologic role in some patients with essential hypertension, i.e., that their hypertension has a neurogenic component. We were surprised to find no relationship between the fall in pressure and the fall in plasma NE after clonidine among the normotensives, since our findings and those of several other groups included a clearcut relationship among the hypertensives. This result, and our finding that the y-intercept for the depressor-change in NE plot among the hypertensives was distinctly above the origin, lead to the suggestion that clonidine may have a second mechanism of anti-hypertensive action besides inhibition of central sympathetic outflow. The combination of a high resting level of plasma NE and a large fall in pressure after clonidine serves to identify patients with essential hypertension in whom increased sympathetic outflow contributes to their high blood pressure.

Proposed Course of Study: The study will be extended to patients with hypertension who are less than 40 years old and to normotensive subjects with a strong family history of hypertension. Clonidine suppression testing may be introduced as part of the routine evaluation of patients with essential hypertension.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01964-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Norepinephrine and isoproterenol kinetics.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R.J. Polinsky	Senior Investigator	OD, NINCDS
	I.J. Kopin	Scientific Director	OD, NINCDS
	C.J. Folio	Clin. Nurse Tech.	OD, NHLBI
	R. Stull	Chemist	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are measuring the kinetics of intravenously infused, tracer-labelled norepinephrine and isoproterenol, in order to determine if abnormalities of overall norepinephrine removal or of neuronal norepinephrine uptake ($uptake_1$) occur in essential hypertension and in orthostatic hypotension.

457

Project Description:

Purpose of Project: We have measured norepinephrine (NE) and isoproterenol (I) kinetics in patients with essential hypertension, normotensive control subjects, and patients with orthostatic hypotensive syndromes, in order to determine if abnormalities in the disappearance of these substances from circulating plasma indicate malfunctions of the sympathetic nervous system or neuronal NE uptake.

Methods: Tracer-labelled ^{3}H-l-NE, ^{14}C-d-NE, and ^{3}H-d,l-I were infused simultaneously into 22 patients with essential hypertension and 13 normotensive control subjects, and kinetics of disappearance of these substances from plasma after a 20 min infusion was examined. Similar infusions occurred in patients with idiopathic orthostatic hypotension and with multiple system atrophy. Simultaneous arm arterial and venous sampling and infusions conducted after pre-treatment with the blocker of neuronal NE uptake, desipramine, are being used to quantify the proportion of NE removal in the arm due to neuronal uptake.

Results and Their Significance: Preinfusion levels of plasma NE were significantly positively correlated with calculated NE spillover rates in both the hypertensive and normotensive groups, but not with NE clearances. The d:l ratio in plasma NE was the same as in the infusate during and after the infusion, even after pretreatment with the neuronal NE uptake blocker, desipramine. Because I is not taken up by nerve endings, the ratio of tritium in I to that in NE increased after the infusion ended. This increase was almost completely abolished by pretreatment with desipramine. The results indicate that (a) increased plasma NE levels seen in some patients with essential hypertension result from increased sympathetic neural activity and not from decreased clearance of NE; (b) changes in the I:NE ratio after simultaneous infusion of both provide an index of neuronal NE uptake in man; and (c) neuronal norepinephrine removal is not stereospecific.

Patients with idiopathic orthostatic hypotension had significantly lower baseline plasma NE levels than either normotensive controls or patients with multiple system atrophy. This decreased average level of plasma NE was due to decreased spillover, not accelerated NE clearance. In patients with multiple system atrophy and in normotensive controls, the disappearance of I from plasma was slower than the disappearance of NE; but in patients with idiopathic orthostatic hypotension, the I and NE disappearance rates were the same. These results suggest that patients with idiopathic orthostatic hypotension have decreased sympathetically mediated NE release from nerve terminals at rest and have deficient neuronal NE removal.

When disappearance kinetics of I and NE were compared in brachial arterial and antecubital venous plasma, the following preliminary results were obtained: (1) The steady-state level of radioactivity in NE in the artery was about twice as high as in the vein, indicating that the arm removes about a half of NE

reaching it. Consequently, the arm contributes importantly to the NE concentration in the antecubital vein. (2) The removal of I in the arm was slightly but consistently less than the removal of NE. This suggests that NE is removed to a small degree by neuronal uptake in the arm, since I is not a substrate for $Uptake_1$. (3) Consistent with this hypothesis, this excessive removal of NE with respect to I in the arm was abolished in subject's pre-treated with desipramine. We currently are evaluating whether comparison of the kinetics of I and NE in artery and vein can be used to quantify neuronal NE removal in this--as well as other--sympathetically innervated beds. If so, then this methodology can be applied to find out, for instance, if renal sympathetic neural activity and renal $Uptake_1$ are abnormal in patients with essential hypertension.

Proposed Course of Project: The NE kinetics clinical protocol (#80 H 107) has been amended to allow simultaneous measurement of NE and I kinetics from the brachial artery and an antecubital vein, for the reasons discussed above.

Publications:

Goldstein DS, Horwitz D, Keiser HR, Polinsky RJ, Kopin IJ. Plasma l-^{3}H-norepinephrine, d-^{14}C-norepinephrine, and d,l-^{3}H-isoproterenol kinetics in essential hypertension. J Clin Invest 72:1748-1758, 1983.

Goldstein DS, Horwitz D, Keiser HR, Polinsky RJ, Kopin IJ. Plasma l-^{3}H-norepinephrine, d-^{14}C-norepinephrine, and d,l-^{3}H-isoproterenol kinetics in essential hypertension. Proceedings, 5th Catecholamine Symposium, Goteborg, Sweden. New York: Pergamon, 1983, p. 148.

Polinsky RJ, Goldstein DS, Horwitz D, Keiser HR, Kopin IJ. Plasma catecholamine kinetics in patients with chronic autonomic failure and control subjects. Proceedings, 5th Catecholamine Symposium, Goteborg, Sweden. New York: Pergamon, 1983, p. 250.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01965-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Assay procedures for measuring catecholamines.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R. Stull	Chemist	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI
	S. Markey	Pharmacologist	LCS, NIMH
	E. Marks	Guest Worker	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are using liquid chromatography with electrochemical detection to measure free catecholamine and DOPA concentrations in plasma and urine.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Purpose of Project: We have modified the liquid chromatographic-electrochemical assay procedure for catecholamines in order to measure urinary free catecholamine concentrations. This methodology was applied to determine whether plasma and urinary catecholamines change as a function of the stage of the human ovulatory cycle. We also developed a new technique for simultaneously measuring plasma and urinary levels of free DOPA, DOPAC, and the catecholamines. We sought to identify a commonly-occurring contaminating peak in the assay for plasma catecholamines in people.

Methods: Additional sample purification steps with commercially available C-18 and silica pre-packed columns were used prior to alumina batch extraction for urinary catecholamines. Daily blood samples and 24 hour urine collections were obtained from 6 healthy inpatient volunteers over the course of an entire ovulatory cycle. We modified the chromatographic conditions, used a more sensitive electrochemical detector which included series oxidizing and reducing electrodes, and used an acetonitrile gradient to separate and quantify DOPA, DOPAC, and the catecholamines. The unknown peak was identified by co-chromatography with known standards and confirmed by GC-MS.

Results and Their Significance: The modified sample purification steps resulted in excellent separation and quantification of urinary catecholamines. Plasma norepinephrine was at its lowest during the follicular phase of the ovulatory cycle. Plasma norepinephrine began to increase about 2 days before ovulation and continued to increase after ovulation. Urinary norepinephrine excretion showed a similar but attenuated pattern. The DOPA/DOPAC/catecholamine procedure produced excellent separation and quantification of these substances. Of 108 people whose plasma was assayed for catecholamine content using HPLC-ED, 36 (33%) had an additional peak larger than that of NE, E, or dopamine and with a retention time between that of E and dopamine. We found that this peak was due to dihydrocaffeic acid (DHCA), which is a catechol metabolite of caffeic acid.

Addition of sample purification steps with commercially available pre-packed columns prior to alumina batch extraction resulted in excellent chromatographic records, whereas assaying urine after only an alumina extraction yielded unreliable results. The modified technique allows injection of urine-derived eluates into the same HPLC-ED system as for plasma-derived eluates. Cyclic patterning of plasma norepinephrine is one of many factors which should be considered in the design and analysis of studies which use plasma norepinephrine as an indicator of sympathetic neural activity in human disease states. Increased sympathetic activity appears to precede ovulation. Our DOPA/DOPAC/catecholamine procedure is the first successful use of an organic-phase gradient with an electrochemical detector to separate and quantify these substances, and the series of oxidizing and then reducing electrodes has allowed much better sensitivity for measuring plasma levels of epinephrine and dopamine. When HPLC-electrochemical procedures are used to

measure plasma catecholamines in people, dietary factors can produce contaminating peaks and lead to erroneous clinical interpretations. One such factor is DHCA, a metabolite of a coffee-containing catechol.

Proposed Course of Project: We have adopted the modified assay for DOPA, DOPAC, and the catecholamines and will be using this procedure routinely. We will use the modified sample preparation for urinary catecholamine assays. Future studies will proscribe drinking of coffee in any form for at least 18 hours prior to blood sampling. A few studies are in progress which involve the inter-relationships among circulating DOPA, plasma catecholamines, urinary dopamine, and sodium excretion. Dr. Paul Levinson is the Principal Investigator for these studies.

Publications:

Goldstein DS. Modified sample preparation for high-performance liquid chromatographic-electrochemical assay of urinary catecholamines. J Chromatog 275:174-177, 1983.

Goldstein DS, Levinson P, Keiser HR. Plasma and urinary catecholamines during the human ovulatory cycle. Amer J Obstet Gynecol 146:824-829, 1983.

Goldstein DS, Stull R, Zimlichman R, Levinson PD, Smith H, Keiser HR. Simultaneous measurement of DOPA, DOPAC, and catecholamines in plasma by liquid chromatography with electrochemical detection. Clin Chem 30:815-816, 1984.

Goldstein DS, Stull R, Markey SP, Marks E, Keiser HR. Dihydrocaffeic acid: A common contaminant in the liquid chromatographic-electrochemical measurement of plasma catecholamines in man. J Chromatog Biomed Applic (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01966-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Plasma catecholamines as a function of aging and high blood pressure.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	I.J. Kopin	Scientific Director	OD, NINCDS
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS (if any)
USUHS, Bethesda, MD (C.R. Lake, B. Chernow); SUNY, Stony Brook, NY (D.G. Glass); Holt-Crock Dialysis Ctr., Fort Smith, AR (M. Coleman); UCSD, SanDiego, CA (M.G. Ziegler); Dept. Med., Baylor Coll. Med., Houston, TX (A.A. Taylor, J.R. Mitchell).

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)
- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have used plasma catecholamine concentrations to estimate activity of the sympathetic nervous system and its relationship to aging and high blood pressure.

GPO 904-917

Purpose of Project: We assessed the relationship between venous plasma levels of norepinephrine (NE) and subject age in patients with primary or secondary hypertension and in normotensive controls.

Methods: Individual data from several previously published studies were reanalyzed.

Results and Their Significance: Among 129 normotensive subjects, plasma NE increased significantly with subject age, but among hypertensives no age-related increase occurred. When subjects less than 40 years old were considered, hypertensives had significantly higher plasma NE than the controls (317 vs 245 pg/ml, p 0.01); but above the age of 40 years, no significant hypertensive-normotensive difference was obtained. Among patients with secondary hypertension (diabetes mellitus, primary hyperaldosteronism, polycystic kidney disease, bilateral parenchymal renal disease, renal artery stenosis), only the group with renal parenchymal disease had elevated levels of supine plasma NE. Among 41 patients with essential hypertension and 59 normotensive controls, the distributions of NE and epinephrine (E) values were shifted upward in the hypertensives. NE and E levels were uncorrelated.

These results are consistent with the hypothesis that increased sympathetic neural activity occurs in the early stages of essential hypertension. No abnormality of plasma NE is apparent in secondary forms of clinical hypertension with the exception of bilateral renal parenchymal disease, where defective NE clearance, rather than excessive sympathetically mediated NE release, may cause high levels of resting plasma NE. Abnormalities of NE and E appear to be independent in essential hypertension.

Proposed Course of Project: These studies are completed.

Publications:

Goldstein DS, Lake CR, Chernow B, Ziegler MG, Coleman MD, Taylor AA, Mitchell JR, Kopin IJ, Keiser HR. Age-dependence of hypertensive-normotensive differences in plasma norepinephrine. Hypertension 5:100-104, 1983.

Lake CR, Chernow B, Goldstein DS, Glass DG, Coleman M, Ziegler MG. Plasma catecholamine levels in normal subjects and in patients with secondary hypertension. Fed Proc 43:52-56, 1984.

Goldstein DS, Lake CR. Plasma norepinephrine and epinephrine levels in essential hypertension. Fed Proc 43:57-61, 1984.

Goldstein DS, Lake CR, Ziegler MG. Plasma norepinephrine in essential hypertension. In Ziegler MG, Lake CR (Eds), Norepinephrine: Clinical Aspects, Baltimore, Williams & Wilkins, 1984 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01967-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Baroreflex sensitivity in essential hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	C.J. Folio	Clin. Nurse Tech.	OD, NHLBI
	B. Chidakel	Engineer	BEIB, DRS
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have used several techniques to measure arterial baroreflex sensitivity in essential hypertension and related baroreflex sensitivity to sympathetic activity as indicated by plasma catecholamines.

495

Project Description:

Purpose of Project: We have evaluated baroreflex-cardiac sensitivity and related it to plasma norepinephrine (NE) in patients with essential hypertension and in normotensive control subjects.

Methods: Sensitivity of the cardiac limb of the arterial baroreflex was determined by blood pressure and interbeat interval responses associated with the Valsalva maneuver, externally applied neck suction and pressure, and injection of phenylephrine and nitroglycerin in 25 patients and in 29 control subjects. Pressor responsiveness to injected phenylephrine and levels of catecholamines in arm arterial and venous blood also were assessed. We also evaluated the effects on parasympathetic blockade with atropine (2 mg/70 kg) on these measures in several normotensives.

Results and Their Significance: By all the measurement techniques, patients with essential hypertension had significantly decreased baroreflex sensitivity. Hypertensive patients also had significantly higher mean levels of plasma NE and epinephrine (E) in both brachial arterial and antecubital venous blood (246 vs]54 pg/ml arterial NE, 286 vs 184 pg/ml venous NE, 99 vs 55 pg/ml arterial E, and 65 vs 35 pg/ml venous E) and significantly larger pressor responses to injected phenylephrine. There was also a significant inverse relationship between sensitivity and venous NE and between sensitivity and pressor responsiveness. The results indicate that decreased baroreflex-cardiac sensitivity, increased sympathetic outflow, and pressor hyperresponsiveness tend to occur together in some patients with essential hypertension. Decreased arterial distensibility and altered central neural integration can account for these findings. Atropinization augmented the depressor response during the Valsalva maneuver (269%), the pressor response after release of the maneuver (544%), the pressor response to phenylephrine (109%), and the depressor response to nitroglycerine (76%), whereas the depressor response to externally applied neck suction was attenuated or abolished in all 12 subjects tested. Cardiac output as indicated using impedance cardiography was unchanged by carotid baroreceptor stimulation. The atropine results are best explained by inhibition of compensatory heart rate, vasodilator, and negative inotropic responses.

The obtained inverse relationship between baroreflex-cardiac sensitivity and plasma NE may have resulted from excessive stress responses to the experimental situation in some patients with hypertension. If this is true, then the relationship would be expected to be abolished in sedated subjects. Accordingly, the baroreflex protocol was amended to test the effects of sedation with intravenous diazepam on baroreflex sensitivity and on plasma NE. Preliminary results suggest that sedation does not affect baroreflex-cardiac sensitivity but does cause decreased plasma NE. If confirmed upon further study, this result would be consistent with the hypothesis that high levels of plasma NE in some hypertensives

result from excessive stress responses, but that sedation does not normalize their baroreflex-cardiac sensitivity.

Proposed Course of Project: The project is being extended as noted above. We also plan to evaluate normotensive subjects with a strong family history of essential hypertension, in order to determine if abnormalities of the baroreceptor-cardiac reflex can antedate the development of hypertension.

Publications:

Goldstein DS. Arterial baroreflex sensitivity, plasma catecholamines, and pressor responsiveness in essential hypertension. Circulation 68:234-240, 1983.

Goldstein DS. Keiser HR. Pressor and depressor responses after cholinergic blockade in man. Amer Heart J 107:974-979, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01968-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Stress, pain, and the sympathetic nervous system in hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R. Dionne	Staff Fellow	NAB, NIDR
	P.R. Wirdzek	Nurse Specialist	NAB, NIDR
	W. Maixner	P.R.A.T. Fellow	NAB, NIDR

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are determining whether patients with essential hypertension have excessive sympathetic or circulatory responses to the stress and pain of oral surgery.

498

Project Description:

Objectives: We have studied behavioral, circulatory, and plasma catecholamine responses to dental surgery as a model for a real-life, controllable, environmental stress. We measured the effects of sedation with intravenous diazepam intravenous diazepam and of inclusion of epinephrine in the local anesthetic on the above measures. We also are conducting a study to determine if the previously noted increased pain threshold of patients with essential hypertension is normalized with the opiate antagonist, naloxone.

Methods: In 18 dental outpatients undergoing surgical removal of impacted third molars (35 procedures), circulatory, biochemical, and behavioral measures were obtained before, during, and after wisdom tooth extractions. Subjects were randomly assigned to receive sedation with intravenous diazepam and to receive epinephrine in the local anesthetic.

Results and Their Significance: In unsedated patients, significant elevations in venous plasma epinephrine (E) levels (203% above baseline) and in cardiac output (30%, as indicated using impedance cardiography) occurred after administration of local anesthetic containing E, whereas no changes were seen after the local anesthetic alone. Unsedated subjects had increased plasma norepinephrine (NE, 24%) and E (57%) levels during the surgery. Premedication with diazepam decreased plasma NE by 29% below the baseline pre-operative level, followed by an increase during surgery to about the pre-operative level. Diazepam abolished the E response to the surgery. These results indicate that intraoral injections of epinephrine-containing local anesthetics result in increased circulating levels of E that are associated with cardiovascular changes and that diazepam premedication decreases plasma NE levels and attenuates the sympathoadrenal response to surgical stress.

Proposed Course of Project: The study will be extended to patients with essential hypertension and to normotensive subjects with a strong family history of essential hypertension. Special recruitment efforts will be needed to include these subjects. A clinical protocol has begun in which the NHLBI and NIDR are collaborating in studying pain thresholds in hypertension and the effects of naloxone. Dr. William Maixner is the Principal Investigator for that study.

Publications:

Goldstein DS, Dionne R, Sweet J, Gracely R, Brewer HB Jr, Gregg R, Keiser HR. Circulatory, plasma catecholamine, cortisol, lipid, and psychological responses to a real-life stress (wisdom tooth extractions): Effects of diazepam sedation and of inclusion of epinephrine with local anesthetic. Psychosom Med 44:259-271, 1982.

Dionne RA, Goldstein DS, Wirdzek PR. Effects of diazepam premedication and epinephrine-containing local anesthetic on cardiovascular and plasma catecholamine responses to oral surgery. Anesth Analg 63: ---, 1984 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01969-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chemotherapy for malignant pheochromocytoma.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

PI:	H.R. Keiser	Deputy Chief	HE, NHLBI
Others:	D.S. Goldstein	Senior Investigator	HE, NHLBI
	R. Stull	Chemist	HE, NHLBI
	S. Averbush	Medical Staff Fellow	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have treated three patients with rapidly progressive malignant pheochromocytoma with combination chemotherapy. All three patients have shown significant responses to treatment with objective decreases in tumor size, marked reductions in output of catecholamines and/or their metabolites and in the requirement for antihypertensive drugs and marked improvement in activity level and sense of well being. All subjects continue on chemotherapy and side effects have been minimal.

Project Description:

Objectives: A pheochromocytoma is a rare tumor. Fortunately only 10 to 15% are malignant. However, because the disease is so rare there are few reports about chemotherapy for this disease and those are mainly anecdotal. Therefore we sought to develop a logical basis for a chemotherapeutic regimen for malignant pheochromocytoma.

Methods: Recently we had seen two young men with malignant pheochromocytoma that were growing and metastasizing very rapidly. Yet there was no accepted form of therapy for this disease. The tumors resembled malignant neuroblastomas in that they arise from the same embryonic tissue and produce catecholamines. However, neuroblastomas are much more common tumors and systematic studies have yielded good data on the efficacy of various chemotherapeutic regimens. Therefore we chose a regimen of cyclophosphamide (750 mg/M^2, IV on day 1), vincristine (1.4 mg/M^2, IV on day 2), and dacarbazine (600 mg/M^2 on days 1 and 2) that had been shown to be very effective in treating neuroblastoma. We repeated therapy in 21 day cycles and adjusted the doses of drugs up or down on the basis of hematologic responses and toxicity.

Results: We have now treated three patients with malignant pheochromocytoma. Each patient had life-threatening distant metastases and poorly controlled hypertension inspite of therapy with large doses of both antiadrenergic drugs and an inhibitor of catecholamine synthesis. Each patient had a decrease in blood pressure within the first few cycles of treatment and this correlated with significant reductions in plasma and urinary catecholamines and objective regression of tumor size.

		Patient 1	Patient 2	Patient 3
Urinary catecholamines (ng/24hr)	Pre-Rx	2340	2787	4186
	Post-Rx	227	552	---

Normal value is less than 150.

At a followup of 4 to 11 months each patient continues to receive chemotherapy with stable disease in patient 3 and continuing regression of tumor in patients 1 and 2. In each patient the blood pressure is well controlled with minimal or no antihypertensive therapy. Two patients exhibited acute toxic syndromes following chemotherapy that were most likely due to tumor lysis. There have been few side effects and dose limiting toxicity has been due to myelosuppression and neurotoxicity. Each patient has shown an increased level of activity and a marked improvement in his sense of well being since starting chemotherapy.

Significance: We have seen remarkable improvement in 3 patients with malignant pheochromocytoma given combination chemotherapy. While these are preliminary studies they are the first time any chemotherapeutic program has been shown to be effective in this disease. We are thus greatly encouraged and very optimistic about further use of this therapeutic regimen.

Proposed Course of Study: More patients with malignant pheochromocytoma will be treated as they become available. We will attempt to culture the tumors and to measure various peptide and hormone markers to determine more about the actions of these specific drugs on this type of tumor.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01970-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Neural circulatory control in the hyperdynamic circulatory state syndrome.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We evaluated neural circulatory control mechanisms in a patient with the hyperdynamic circulatory state syndrome.

Project Description:

Purpose of Project: We performed extensive testing of neural circulatory control mechanisms in a patient with the hyperdynamic circulatory state syndrome (resting tachycardia, labile hypertension, increased resting cardiac index, excessive tachycardic response to isoproterenol, and amelioration by propranolol).

Methods: A 22 year old man was admitted to the Clinical Center for a possible pheochromocytoma, which conclusively was excluded. The patient underwent baroreflex testing, a clonidine suppression test, provocative testing with yohimbine and isoproterenol, and treatment with propranolol.

Results and Their Significance: Baroreflex-cardiac sensitivity was remarkably poor in this subject at a time when he had resting tachycardia and high circulating levels of catecholamines. Isoproterenol and yohimbine elicited severe hypertension, tachycardia, flushing of the chest, and anxiety, all of which were immediately reversed by intravenous propranolol. Propranolol treatment also induced as well as spontaneously-occurring acute hypertensive episodes. Since the heart rate limb of the arterial baroreflex depends on changes in vagal outflow, and plasma norepinephrine (NE) levels seem to index sympathetic outflow, this patient had simultaneous parasympathetic inhibition and sympathetic stimulation associated with his hypertension and tachycardia. Neither of these findings can be easily explained by excessive beta-adrenoceptor responsiveness, which has been thought to cause this syndrome. Rather, it appears that an abnormality of central neural circulatory control, exacerbated during anxiety responses, cuased the patient's hyperdynamic circulatory state.

Proposed Course of Project: We will similarly evaluate other patients with this interesting disorder as referrals allow.

Publications:

Goldstein DS, Keiser HR. Neural circulatory control in the hyperdynamic circulatory state syndrome. Amer Heart J (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01971-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Modulation of the brachial arterial dicrotic wave in essential hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R. Chadwick	Biomedical Engineer	BEIB, DRS
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

We recorded modulation of the brachial arterial pressure dicrotic wave, developed a circulatory model to explain this modulation, and related the amount of modulation to arterial plasma norepinephrine in patients with essential hypertension.

Project Description:

Purpose of Project: We developed a circulatory model of the brachial arterial system to predict the main changes in the arterial pressure waveform as it traverses the vasculature and used the model to explain observed modulation of the brachial arterial dicrotic wave in patients with essential hypertension and in normotensive control subjects.

Methods: The model considers the brachial artery as a tapered, distensible tube with side branches and ending in a loop, with amplication of the frequency components of the input flow pulse corresponding to the dicrotic and main waves as they propagate. The amount of vertical modulation of the dicrotic wave with respect to the pulse pressure was measured after phenylephrine or nitroglycerine injection in 27 patients with essential hypertension and in 35 normotensive control subjects. Arterial blood samples were obtained for plasma norepinephrine (NE) determinations.

Results and Their Significance: The human brachial arterial dicrotic wave moves inward and upward with respect to the main wave during phenylephrine-induced vasoconstriction and downward and outward during nitroglycerine-induced vasodilation. These changes, resulting from combined alterations of arterial stiffness and arteriolar caliber, also were predicted by the circulatory model. The amount of modulation of the dicrotic wave decreased with increasing age and with high blood pressure, the model explaining these findings in terms of increased arterial rigidity and decreased arteriolar vasodilator responsiveness. A significant negative correlation between the arterial levels of plasma NE and modulation of the dicrotic wave after nitroglycerine among subjects 40 years old or younger suggested a sympathetic neurogenic contribution to the vascular abnormalities observed in relatively young patients with essential hypertension.

Proposed Course of Project: We currently are refining the circulatory model to predict better the exact shape of the brachial arterial pressure waveform. Methods may be developed to measure arterial rigidity and arm vascular impedance independently to test the model further.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01972-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Renal catecholamine release in dogs.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Marks	Guest Worker	HE, NHLBI
Others:	G. Kelly	Biol. Lab. Tech.	HE, NHLBI
	D.S. Goldstein	Senior Investigator	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We measured the effects of renal artery ligation and renal nerve section on renal release of catecholamines in dogs.

Project Description:

Purpose of Project: We have examined renal catecholamine release in dogs during acute renal artery constriction and after renal nerve section, in order to determine the role of sympathetic outflow in the acute hypertension in this model and to determine the kidney's contribution to circulating plasma norepinephrine (NE).

Methods: In anesthetized dogs, renal arterial clipping and nerve section occurred and the effects on renal flow and urinary, arterial and renal venous NE assessed both in the ipsilateral and contralateral kidney.

Results and Their Significance: All subjects studied so far have had a net arteriovenous increment in plasma NE which was attenuated or abolished both by renal artery clipping and by renal nerve section. These maneuvers also appeared to produce decreased NE release by the contralateral kidney. These preliminary results suggest that the arteriovenous increment in NE in the kidney reflects sympathetic innervation to that bed. The effects on the contralateral kidney may be consistent with the existence of a reno-renal reflex influencing sympathetic outflow to the contralateral kidney.

Proposed Course of Project: Unfortunately, limitations on staff time for this project have precluded completion of this project. We hope to continue to assess the effects of renal artery clipping, renal nerve section, and renal nerve stimulation (proximal and distal ends after nerve section) on the ipsilateral and contralateral release of NE.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01973-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Circulating DOPA is a source of urinary dopamine in dogs.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.D. Levinson	Senior Staff Fellow	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI
	D.S. Goldstein	Senior Investigator	HE, NHLBI
	G. Kelly	Biol. Laboratory Tech.	HE, NHLBI
	T. Forrester	Visiting Fellow	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.5	.5

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although the kidney is an active site of conversion of DOPA to dopamine (DA), the amount of circulating DOPA which is converted to DA in large animals is not known. Using a radiotracer technique in dogs, preliminary studies indicate that approximately 10% of free urinary DA is derived from renal decarboxylation of circulating DOPA.

Project Description:

Objectives: Dopamine may modulate sodium excretion in two ways: either directly at the kidney level, or indirectly through an effect on aldosterone release. We assessed the dopamine-mediated contribution of aldosterone by analyzing the natriuretic responses to saline in normal and hypoadrenal subjects, before and after administration of the dopamine-antagonist, metoclopramide.

Methods: Normal volunteers and patients with documented primary adrenal insufficiency were studied after achieving sodium balance on a 109 mEq/day sodium intake. Two liters of normal saline were infused over a 4-hour period on two occasions at least 6 days apart. Metoclopramide was administered in a randomized manner on one of the two test days, in a .075 ng/kg bolus, followed by a 0.15 ng/kg infusion for 4 hours.

Results: Eight normal volunteers and two subjects with adrenal insufficiency completed the study. Most of the subjects noted mild to moderate symptoms of restlessness and/or sedation during metoclopramide administration. Plasma prolactin increased in all subjects after metoclopramide, while plasma aldosterone increased in 5 of 8 subjects. Fractional excretion of sodium decreased markedly in four subjects. The change in fractional excretion of sodium showed a correlation of 0.73 ($p<.05$) relative to the change in aldosterone, and 0.37 (p=NS) relative to the change in plasma metoclopramide levels. One of the two patients with adrenal insufficiency showed a decrease in fractional excretion of sodium during metoclopramide; the other did not.

Significance: The significant correlation between the metoclopramide-induced rise in aldosterone and reduction in sodium excretion suggests that the anti-natriuretic effect of the dopamine-antagonist is mediated by its stimulation of aldosterone secretion from the adrenal glomerulosa. Further studies in patients with adrenal insufficiency should validate this conclusion.

Proposed Course of Study: Two to four additional subjects with primary adrenal insufficiency will be studied.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01974-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of metoclopramide on saline-induced natriuresis in man.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.D. Levinson	Senior Staff Fellow	HE, NHLBI
Others:	D. S. Goldstein	Senior Investigator	HE, NHLBI
	J. R. Gill, Jr.	Senior Investigator	HE, NHLBI
	H. R. Keiser	Deputy Chief	HE, NHLBI
	J. Folio	Clin. Nurse Tech.	OD, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Endogenous dopamine may modulate sodium metabolism through either an aldosterone-inhibiting effect, or a renal vascular or tubular natriuretic effect. Preliminary data indicate that the anti-natriuresis produced by the dopamine-antagonist, metoclopramide, is mediated predominantly by stimulation of aldosterone secretion from the adrenal zona glomerulosa.

3 / /

Project Description:

Objectives: It has been estimated that 30% of urinary dopamine (DA) in the rat is derived from circulating 3,4-dihydroxyphenylalamine (DOPA); estimates from clearance studies in man and dogs range from 30 to 90%. We proposed to more accurately analyze the intra-renal metabolism of circulating DOPA by using a radioactive tracer technique.

Methods: One hundred microcuries of ^{3}H-DOPA were infused into the left renal artery of 2 anesthetized dogs during a water diuresis. Arterial and left renal venous plasma, and urine from both ureters were analyzed for DOPA and DA concentration and radioactivity.

Results: The preliminary data indicate that a maximum of 10 to 15% of urinary dopamine is derived from circulating DOPA. Since the filtered load of DOPA is about 120 ng/min, >95% of the DOPA is either reabsorbed or metabolized to other compounds.

Significance: Urinary dopamine usually changes in parallel with sodium excretion, and dopaminergic mechanisms for modulating sodium excretion have been postulated. This study and further investigations under varied conditions of salt-intake will help to define the sources of urinary dopamine, and dopamine's role as a possible natriuretic factor in the kidney.

Proposed Course of Study: Four to six more animals will be studied under this protocol.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01975-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of adrenergic system in vasoconstriction by acetylcholine in dogs.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Yun	Guest Worker	HE, NHLBI
Others:	J.R. Gill, Jr.	Senior Investigator	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of the adrenergic nervous system in the renal vasoconstriction by acetylcholine (ACh) in indomethacin (INDO)-treated dogs was examined in dogs pretreated with reserpine (R) or in dogs receiving an intrarenal infusion of phenoxybenzamine (P). In INDO-treated dogs which also received treatment of R (0.5 mg/kg/day, I.M. for 2 days) or P (0.5 mg/kg followed by 190 µg/min), renal arterial infusion of ACh produced an initial rise in sodium excretion (UNaV) and renal plasma flow (RPF) with no change in renin secretion (RS). This was followed by a fall in UNaV and RPF with an increase in RS. It is concluded that ACh produces renal vasoconstriction in INDO-treated dogs by mechanism(s) other than an increase in the release of norepinephrine from renal sympathetic nerves.

Project Description: Z01 HL 01975-01 HE

Objectives: Infusion of acetylcholine (ACh) into a renal artery of the dog is known to cause an increase in renal plasma flow (RPF) and sodium excretion ($U_{Na}V$). How ACh produces renal vasodilation and natriuresis is not clear. Recently, we have found that the synthesis of prostaglandins is required for the vasodilatory and natriuretic response to ACh. In that study renal arterial infusion of ACh in control dogs produced a sustained increase in RPF and $U_{Na}V$ without a change in glomerular filtration rate (GFR) or renin secretion rate (RSR). In dogs pretreated with indomethacin (INDO), an inhibitor of PG synthetase, renal arterial infusion of ACh produced an initial increase and then a decline in RPF and $U_{Na}V$ that was accompanied by a progressive fall in GFR, and a progressive rise in RSR. The present series of experiments was to determine whether pretreatment of the dog with reserpine or renal arterial infusion of phenoxybenzamine will prevent the renal vasoconstriction by ACh in INDO-treated dogs.

Methods: Mongrel dogs were given a diet containing 180 mEq/day of sodium for one week before study. On the morning of the experiment, the dogs were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and ventilated with a respirator. Tygon catheters were placed in a femoral artery for the collection of arterial blood samples and measurement of blood pressure and in a femoral and a jugular vein for infusion.

The abdomen was opened through a mid-line incision and tygon catheters were inserted into both ureters for collection of urine and into the left renal vein via the left ovarian vein for the collection of renal venous blood samples. A 20-gauge needle connected to a tygon catheter was inserted into the left renal artery for infusion into the left kidney. A flow probe (Micron RC 1000) was placed on the left renal artery for measurement of renal blood flow (RBF).

One thousand ml of lactated Ringer solution were given during the operative procedures followed by an infusion of lactated Ringer solution at 5.2 ml/min. Inulin 30 mg/kg dissolved in saline was given as a primer, followed by a sustaining infusion at 0.58 ml/min to maintain a plasma concentration of approximately 20 mg/100 ml. Urine was collected at 20 minute intervals and, at the mid-point of each collection, arterial (15 ml) and left renal venous (5 ml) blood samples were drawn. When a sample was taken, an equal amount of blood from donor dogs was given as replacement.

After the surgical procedures had been completed, normal saline was infused into the left renal artery at 0.1 ml/min. When urine flow rate had stabilized, the dogs were then studied according to the following protocols:

Group I: Intrarenal infusion of ACh (7 dogs) - After three consecutive 20-minute control clearance periods were obtained, ACh was then infused into the left renal artery at 40 µg/min for five 20-minute periods. Left renal arterial infusion of saline was then resumed for two 20-minute post-control periods.

Group II: Intravenous injection of indomethacin plus intrarenal infusion of ACh (10 dogs) - After a 20-minute control clearance period, a bolus of

indomethacin (5 mg/kg) was given intravenously and two 20-minute clearance periods were obtained. ACh was then infused into the left renal artery at 40 µg/min for five 20-minute periods. Saline was then infused into the left renal artery for two 20-minute post-control periods.

Group III: Intravenous injection of indomethacin plus intrarenal infusion of phenoxybenzamine and ACh (7 dogs) - A bolus of indomethacin (5 mg/kg) was given intravenously and thirty minutes later a clearance period was obtained. An alpha receptor blocker, phenoxybenzamine, was then infused into the left renal artery (0.5 mg/kg, followed by 190 µg/min). (In preliminary experiments, this dose of phenoxybenzamine has been shown to effectively prevent the vasoconstriction by intrarenal injection of norepinephrine). Phenoxybenzamine was infused for nine 20-minute periods. After phenoxybenzamine was infused for two periods, ACh (40 µg/min) was then added to the perfusate for five periods.

Major Findings and Significance: Renal arterial infusion of ACh produces a natriuresis and an increase in renal plasma flow without a change in glomerular filtration rate (GFR) or renin secretory rate (RSR). In dogs pretreated with INDO, an inhibitor of prostaglandin synthesis, renal arterial infusion of ACh produced an initial increase followed by a decrease in sodium excretion, RPF, and GFR, and a progressive increase in RSR. The decline in sodium excretion, RPF, and GFR, and the rise in RSR with ACh in INDO-treated dogs could not be prevented by prior treatment with reserpine or intrarenal infusion of phenoxybenzamine (Table I). The present findings suggest that ACh produces renal vasoconstriction in INDO-treated dogs by mechanism(s) other than an increase in the catecholamine release from renal sympathetic nerve endings.

Proposed Course of Study: The mechanism(s) by which ACh produces renal vasoconstriction in INDO-treated dogs remains unclear. It is possible that ACh causes an increase in the influx of calcium from the extracellular fluid space into intracellular fluid space in INDO-treated dogs. If this is so, pretreatment with calcium-entry blockers, such as verapamil, would be expected to prevent the vasoconstriction by ACh in INDO-treated dogs. This possibility is currently being examined.

Publications:

Yun, J.C.H., Gill, J.R., Jr., Ho, S.S., Kelly, G.D. and Keiser, H.R.: Prostaglandin E_2 but not $F_{2\alpha}$ restores the natriuretic response to acetylcholine in indomethacin-treated dogs. Am. J. Physiol. 247: F185 - F191, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01976-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
Effect of sodium intake on calcium metabolism in salt-sensitive hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name title, laboratory and institute affiliation)*

PI:	J.R. Gill, Jr.	Senior Investigator	HE, NHLBI
Others:	H.-G. Gullner	Guest Worker	HE, NHLBI

COOPERATING UNITS *(if any)*
Department of Psychiatry, USUHS, Bethesda, MD (C.R. Lake);
Department of Pathology, St. Paul-Ramsey Medical Center, St. Paul, MN (D.J. Lakatua)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Previous studies have identified two subsets of essential hypertensives, those whose blood pressure increased when sodium intake was increased from 9 to 249 mEq/day (salt-sensitive) and those whose blood pressure did not change (salt-resistant). The salt-sensitive patients also showed more sodium retention and weight gain before coming into sodium balance than did the salt resistant. In preliminary studies of calcium and magnesium metabolism salt-sensitive hypertension, an increase in sodium intake from 9 to 249 mEq/day was associated with an increase in urinary calcium from 8.5 to 19.3 mEq/day whereas urinary magnesium showed little change (7.4 versus 8.4 mEq/day). These findings suggest that the greater sodium retention in the salt-sensitive hypertensives may be associated with an increase in calcium excretion. The role of calcium loss in the associated rise in blood pressure remains to be determined.

Project Description:

Objectives: Previous studies indicate that patients with essential hypertension may be classified as salt-sensitive or non-salt-sensitive, depending on whether blood pressure increases or remains unchanged when sodium intake is increased from 9 to 249 mEq/day. The salt-sensitive patients retained more sodium and gained more weight before coming into sodium balance. The salt-sensitive patients also showed a higher plasma and urinary norepinephrine during the high sodium intake than did the salt-resistant patients. Because a number of reports have suggested that calcium metabolism may be abnormal in essential hypertension and may be a factor in its pathogenesis, the present studies were initiated to examine calcium metabolism in salt-sensitive and salt-resistant hypertension.

Methods: Patients with salt-sensitive and non-salt-sensitive hypertension were requested to stop all medications for two weeks before study. They were admitted to an air-conditioned unit in the Clinical Center and were fed a constant metabolic diet containing 9 mEq/day of sodium with 100 mEq of supplemental sodium as sodium chloride for seven days. Sodium intake was then decreased to 9 mEq/day for seven days followed by an increase to 240 mEq/d for eight days. Calcium and magnesium intakes remained the same throughout the study. All urine was collected throughout the studies and analyzed for sodium, potassium, magnesium and calcium. Plasma for sodium, potassium, calcium and magnesium was collected every other day throughout the study.

Results: In salt-sensitive hypertension, mean urinary calcium and magnesium were 16.8 and 8.85 mEq/d, respectively, during a sodium intake of 109 mEq/d and decreased to 8.5 and 7.4 mEq/d, respectively by the end of the seven days of low sodium intake. When sodium intake was increased to 249 mEq/d urinary calcium increased to 18.5 mEq/d while magnesium remained unchanged at 7.8 mEq/d. Serum calcium and magnesium were unchanged throughout. Blood pressure decreased from 122 to 110 mmHg when sodium intake was decreased from 109 to 9 mEq/day then rose again to 120 mmHg when sodium intake was increased to 249 mEq/d. These results suggest that an unrestricted sodium intake can increase calcium excretion and may produce a negative calcium balance in patients with salt-sensitive hypertension. A negative calcium balance may constitute an additional abnormality contributing to the increase in blood pressure that also occurs.

Proposed Course of Study: The studies will be continued to determine how sodium intake affects calcium metabolism in salt-sensitive and salt-resistant hypertensives. Measurement of plasma parathyroid hormone and 1,25dihydroxy-vitamin D will also be performed to determine if the negative calcium balance alters these regulators of calcium metabolism in salt-sensitive hypertension.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01977-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Use of the Quin 2 method for measurement of free intracellular calcium.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Zimlichman	Visiting Associate	HE, NHLBI
Others:	S. Zimlichman	Guest Worker	HE, NHLBI
	D. Goldstein	Senior Investigator	HE, NHLBI
	H. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*
Laboratory of Cell Biology and Genetics, NIADDKD, NIH (H. Pollard)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Quin 2 is an EGTA derivative which can be loaded into cells to permit measurement of intracellular free ionic calcium levels, based upon spectrofluorimetric changes when calcium ions are bound. We have applied the method (which was originally described by Tsien et al) to measure free intracellular calcium levels in lymphocytes neutrophils and platelets from peripheral blood, splenocytes, thymocytes and bovine adrenal cells.

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Project Description:

Objectives: To set up and validate in our laboratory a method for the measurement of intracellular free calcium content in a wide range of both large and small cells. We sought to do this so that we could: 1) evaluate the mechanism of action of certain anti-hypertensive drugs, 2) study the role of calcium in the release of catecholamines from adrenal medullary cells, and 3) evaluate recent reports which indicate there is a high degree of correlation between a person's blood pressure and the levels of cytosolic free calcium in his platelets.

Methods: We used the Quin 2 method originally described by Tsien et al. Cells are loaded with an ester of Quin 2, an EGTA derivative that rapidly enters the cell. Once inside, the Quin 2 is de-esterified, can no longer leave the cell, and binds to free calcium. Excess Quin 2 ester is washed away and the resultant changes in the fluorescence of the Quin 2 provide a measurement of the level of free calcium in the cell. We have explored the applicability of this method to lymphocytes, neutrophils and platelets from peripheral blood, to thymocytes and splenocytes from rats, and to bovine adrenal medullary cells.

Results: We have set up the method in our laboratory and we can make it work easily in lymphocytes and adrenal cells with good reliability and reproducibility. In lymphocytes and adrenal cells, the normal free intracellular calcium content is 100 nM. This is the same value reported by others using this method. In platelets the results are more variable and less certain since the platelets must be isolated in calcium-free medium and later incubated with calcium so that re-equilibration can occur. Further studies of better means of isolating and studying the platelets are underway.

The level of free ionic calcium in resting lymphocytes is unchanged by incubation in 10^{-3} M. ouabain. However, if calcium is washed out of lymphocytes and then added back in the presence of ouabain, the calcium enters more quickly and rises to a higher final concentration. The use of a potassium-free incubation medium causes a further increase in free intracellular calcium content.

We have been able to isolate and study bovine adrenal medullary cells with the help of a number of people in the laboratory of Dr. Harvey Pollard. We have used a number of agents to either stimulate or inhibit catecholamine release from these cells and we have used the Quin 2 method to study the relationship between the resultant intracellular free calcium content and the amount of catecholamines released. Our preliminary results show that this relationship is not linear and therefore more complex than previously reported.

Significance: We have set up successfully in our laboratory the Quin 2 method for measurement of free intracellular calcium content in a number of different cell types. We have shown that partial blockade of the Na^+-K^+ ATPase pump by ouabain or an external medium free of potassium causes a more rapid influx of calcium into the calcium-depleted cell and a higher final level of free

calcium. This result is to be expected since the action of this pump is necessary to push calcium out of the cell. We have found that propranolol added to the medium also causes a faster influx of calcium and a 50% increase in final free intracellular calcium content of lymphocytes. This is opposite to what F. Buhler has reported recently in platelets of hypertensive patients treated with propranolol.

Proposed Course of Study: We shall continue to apply these techniques to learn more about the role of intracellular free calcium in: 1) the actions of certain antihypertensive drugs, 2) the release of catecholamines by adrenal medullary cells, and 3) the control of vascular resistance.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01978-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Calcium channel blockade and norepinephrine release.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	T. Ropchak	Biologist	HE, NHLBI
	R. Stull	Chemist	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI
	R. Zimlichman	Visiting Association	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are determining whether blockade of calcium channels affects the release of norepinephrine from vas deferens and vascular tissue.

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Project Description:

Purpose of Project: We have attempted to determine if calcium channel blockade inhibits release of norepinephrine (NE) from sympathetic nerve endings in vas deferens and aortic tissue in rats.

Methods: Tissue freshly removed from the subject is placed in a bath containing pargyline, tyrosine, and desipramine and is electrically stimulated to record physiologic effects and effects on release of NE related to calcium channel blockade.

Results and Their Significance: Calcium channel blockade with nifedipine decreased electrically-induced contractions of both vas deferens and aortic tissue. Measuring NE release proved difficult, because the use of physiologic pH, 37^{o}C bath, and bubbled oxygen almost immediately oxidized released NE. Addition of several anti-oxidants to the bath was not satisfactory. Tissue simply placed in the bath, without oxygenation and without electrical stimulation, showed spontaneous release of NE into the bath, and this release persisted for at least 60 mins. Nifedipine did not appear to decrease this release. However, a significant proportion of released NE apparently wasoxidized, and this introduced a large amount of variability to the data.

Proposed Course of Project: We are considering changing the experimental design by pre-incubating tissue in tritiated NE or tyrosine and assessing the amount of radioactivity released into the bath upon electrical or chemical stimulation before and after calcium channel blockade. Use of anti-oxidants has either interfered with the HPLC quantification of catecholamines or with the physiologic studies, and so we doubt that any anti-oxidant will completely prevent oxidation of released NE. As a result, use of tissue pre-incubated with labelled NE or labelled precursor seems the logical choice.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01979-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of the kallikrein-kinin system in synaptic transmission.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Daniel Goldstein	Expert	HE, NHLBI
Others:	T. Ropchak	Biologist	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have shown that kinin peptides are produced in the gut of the guinea pig and that these peptides oppose the inhibitory action of opiates by enhancing the release of acetylcholine. The output of kinins by the gut is inhibited by aminoglycoside antibiotics that have been shown by others to inhibit kallikrein, the enzyme that forms kinins.

Project Description: Z01 HL 01979-01 HE

Objectives: We have shown previously that kinin peptides release acetylcholine and antagonize the effects of opiates in the longitudinal muscle-myenteric plexus (LMMP) preparation of the guinea pig intestine. We have also shown that aminoglycoside antibiotics (AGA) and amiloride, a potassium-sparing diuretic, block the electrically induced contraction of that preparation. To explore the possible meaning of this bradykinin-acetylcholine interaction we: 1) measured the output of kinins by the tissue after pretreatment with AGA and 2) studied the effects of trasylol and several trisamidine inhibitors of kallikrein, and of captopril, an inhibitor of kininase II, on the LMMP preparation.

Methods: We used the LMMP preparation as described by WDM Paton and MA Zar. To look for kinin output by the preparation we took half of the entire intestine instead of only 4 cm. and incubated it for 15 minutes in buffer with electrical stimulation and with leucyl-leucine, captopril and Bestatin, as preservatives of kinins. Kinin peptides were quantitated by a radioimmunoassay developed previously in our laboratory.

Results: Streptomycin, a typical AGA, inhibited reversibly the LMMP at an ID_{50} of 10^{-3}M. The streptomycin-treated preparation still responded normally to acetylcholine. This indicated that AGA was not toxic to the preparation and did not block acetylcholine receptors. RIA-measurable levels of bradykinin were produced by the preparation, i.e., 200 fg/mg.tissue/minute and these levels were reduced about 67% by AGA treatment. Trasylol (10^{-4}M.), instead of inhibiting electrically induced contractions, caused a 40-50% enhancement. Two tris-amidine type kallikrein inhibitors, IRT-11 and IRT-36, also enhanced the contractions at doses of 10^{-4}, instead of inhibiting them. On the other hand, captopril inhibited the contractions instead of enhancing them. Amiloride, a potassium-sparing diuretic with weak kallikrein inhibitory action, also enhanced the contractions instead of inhibiting them. We then ran a series of experiments to evaluate the effect of serotonin and its analogs on the LMMP preparation. Serotonin enhances the contractions at doses as low as 10^{-8}M. Melatonin has no effect even at doses of 10^{-5}M. Harmine (10^{-5}M) reversibly inhibited contractions while methylsergide (10^{-4}) was toxic to the preparation and its effects were irreversible.

Conclusions and Significance: In the guinea pig LMMP preparation, kinin peptides oppose the inhibitory action of opiates by enhancing acetylcholine release. We now have evidence that kinins are produced in the guinea pig gut and that the output of kinins by the gut can be inhibited by aminoglycoside antibiotics that inhibit kallikrein, the enzyme that forms kinins. Thus we have for the first time a role for kinins in modulating the contractions of the intestine.

We also have evidence that a series of diverse substances can enhance contractions in the LMMP preparation, i.e., trasylol, tris-amidines, and amiloride. The only feature that all these substances have in common is the ability to inhibit serine-proteases, including kallikrein. Since their action to enhance contractions is opposite to what we would expect from an inhibition of kallikrein we must assume that they are acting in some other, as yet unknown, way.

Proposed Course of Project: We shall evaluate the effect of these serine-protease inhibitors on the release of endogenous kinins and acetylcholine by the LMMP preparation.

Publications:

Goldstein, D.S., Ropchak, T.G., Keiser, H.R., Atta, G.J., Argiolas, A., and Pisano, J.J.: Bradykinin reverses the effect of opiates in the gut by enhancing acetylcholine release. J. Biol. Chem. 258: 12122-12124, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01980-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Aldosterone-stimulation by fragments of proopiomelanocortin (POMC).

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H.-G. Gullner	Guest Worker	
Others:	J.R. Gill, Jr.	Senior Staff Investigator	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have examined several peptides derived from pituitary proopiomelanocortin for their effects on cortisol and aldosterone secretion in the autoperfused dog adrenal glands. Synthetic human β-lipotropin [β-LPH-(1-91)], which is released from the pituitary gland concomitantly with ACTH, produced a significant increase in aldosterone, but not cortisol secretion. The time-course of the increase in aldosterone was similar to that previously described for β-endorphin [β-EP or β-LPH-(61-91)], but the dose of β-LPH needed to produce an equipotent response was larger.

Synthetic γ-lipotropin or synthetic β-melanotropin (β-MSH), the C-terminal portion of γ-lipotropin [γ-LPH or β-LPH-(1-58], had no effect on either cortisol or aldosterone secretion. Furthermore, α-EP [α-endorphin, β-LPH (61-76)] or γ-EP [γ-endorphin, β-LPH (61-77)] did not affect the secretion of either adrenal steroid.

Taken together, these data indicate that 1) β-endorphin is the only β-LPH-derived peptide that selectively stimulates aldosterone production in vivo; and 2) the aldosterone-secreting activity is contained in the C-terminal sequence of β-EP.

Project Description:

Objective: We have previously reported that β-endorphin (β-EP) selectively stimulates aldosterone secretion in in vivo perfused dog adrenal glands. The present experiments were carried out to determine whether this action is specific for β-EP or whether other peptides derived from opiomelanocortin have a similar effect on the adrenal cortex. These observations may have clinical relevance because recent evidence suggests that an aldosterone stimulating factor derived from the pituitary may be involved in the pathogenesis of idiopathic aldosteronism.

Methods: The effects on aldosterone and cortisol secretion of naturally occurring peptides derived from β-lipotropin (β-LPH), as well as α-melanotropin (α-MSH) were examined. All peptides were synthetic and their amino-acid sequences were those of the peptides occurring in man. The adrenal glands of female dogs (NIH foxhounds) were isolated and a Hilton pouch was constructed. The pituitary gland and both kidneys were removed to eliminate circulating ACTH and angiotensin II, respectively. After a one-hour control period, the peptides were infused for 50 minutes at a constant rate followed by a one-hour post-control period. Cortisol and aldosterone concentrations of adrenal vein blood were determined by radioimmunoassay and blood flow rate was used to calculate secretion rates of the hormones.

The following peptides were examined: β-lipotropin, γ-lipotropin, β-melanotropin (β-MSH), α-endorphin (α-EP), γ-endorphin (γ-EP), β-endorphin (1-27), and α-melanotropin (α-MSH). It should be noted that considerable evidence suggests that β-MSH is not a naturally occurring peptide but an in vitro artefact.

Results: Of the peptides listed above only β-lipotropin and β-endorphin (1-27) stimulated aldosterone secretion. None of the peptides listed had an effect on cortisol. The data indicate that the aldosterone-stimulating moiety of β-lipotropin is located within β-endorphin. Since α- and γ-endorphin (1-27) had no effect and since β-endorphin (1-27) stimulated aldosterone with the same potency as β-EP (1-31), it can be concluded that the active, aldosterone-stimulating core is contained in the C-terminal sequence of β-EP.

Conclusions and Significance: We have previously shown that the dose of β-EP needed to cause a significant stimulation of aldosterone secretion is within the physiological range, and that β-EP may play a role in the control of aldosterone secretion. The present data support this suggestion and suggest that β-EP is the only β-lipotropin-derived peptide possessing this unique function. β-EP may be the previously proposed pituitary aldosterone-stimulating factor, or, alternatively, one of several factors. Although α-MSH has previously been reported to stimulate aldosterone production in vivo, we observed no effect of α-MSH on either aldosterone or cortisol secretion.

Proposed Course of Study: The determination of the effects of the N-terminal (1-76) fragment of human proopiomelanocortin (POMC) on adrenal cortical steroid production remains to be done in order to characterize completely the activities of the POMC-derived peptides. Such work is presently in progress.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01981-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biogenesis of Leu-enkephalin in brain.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Zamir	Visiting Fellow	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu;LE) is an endogenous opioid peptide that can arise from two distinct precursors: proenkephalin and prodynorphin. Proenkephalin contains four copies of methionine-enkephalin (ME) and one copy each of LE, ME-Arg-Gly-Leu and ME-Arg-Phe. Prodynorphin contains three LE sequences which comprise the N-termini of α-neo-endorphin, dynorphin A and dynorphin B.

Experiments were designed to differentiate LE derived from proenkephalin versus that derived from prodynorphin. The most dense collections of dynorphin-positive fibers and terminals are in the substantia nigra, hippocampus (mossy fibers in regions Ca_3Ca_4) and posterior pituitary, areas rich in dynorphin-related peptides. The concentration of LE in these three regions is significantly higher than that of ME-Arg-Gly-Leu; the ratio of LE to ME-Arg-Gly-Leu is therefore greater than that found in the proenkephalin precursor which is unity. Globus pallidus deafferentation resulted in a significant decrease of dynorphin B and LE, but not ME-Arg-Gly-Leu, in the substantia nigra. Mild intermittent foot shock (0.2 mA, 20 min) causes a significant increase (approximately 25%) of dynorphin B and LE in the substantia nigra, but has no effect on ME-Arg-Gly-Leu concentrations. Thus, in the substantia nigra LE may be derived primarily from prodynorphin. Likewise, in the posterior pituitary, osmotic stimulus (e.g., 2% NaCl as drinking water) causes marked depletion in dynorphin and LE but has no effect on ME levels suggest that in the posterior pituitary LE is derived primarily from dynorphin.

Project Description:

Objectives: The amino acid sequence of the opioid peptide leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu, LE) is found within several larger peptides, which are generated from the precursors proenkephalin and prodynorphin. Proenkephalin contains four copies of methionine-enkephalin (Tyr-Gly-Gly-Phe-ME, ME) and one copy each of LE, ME-Arg-Phe and ME-Arg-Gly-Leu. Prodynorphin contains three LE sequences which comprise the N-termini of α-neo-endorphin, dynorphin A and dynorphin B. Different cells seem to process the enkephalin precursors in different ways. For example, chromaffin cells of the adrenal medulla make and store substantial amounts of high molecular weight enkephalin precursors as well as enkephalin octa-and heptapeptides. In the brain, on the other hand, most of the enkephalin measured in radioimmunoassays is present as ME, ME-Arg-Phe and ME-Arg-Gly-Leu. There is little or no high molecular-weight enkephalin-immunoreactive material. Thus, the proenkephalin molecule can give rise to a variety of cellular secretory products in different tissues depending on the extent and pattern of its intracellular processing.

Prodynorphin-derived peptides are abundant in the neural lobe and throughout the brain but are present in low amounts in the adrenal gland. Although prodynorphin contains three LE sequences, it has been argued that LE derives exclusively from the single copy of LE found in the proenkephalin precursor. This is because the Molar ratio of ME:LE in various brain areas approximately 4:1 roughly correspond to the ratio found in the proenkephalin precursor and because the regional distribution of dynorphin and enkephalin (LE or ME) in the brain differ. There is little information on the processing of prodynorphin to yield LE. The most dense collections of dynorphin-positive fibers and terminals in the rat are in the substantia nigra and neural lobe of the pituitary gland. These areas also contain proenkephalin nerve fibers and terminals.

Experiments were designed to differentiate LE derived from proenkephalin versus that derived from prodynorphin.

Methods: Male Sprague-Dawley rats (200-250g) were killed by decapitation and their brains removed. Tissue samples were microdissected after inspection of the lesions. The posterior lobe of the pituitary gland was isolated under a dissecting microscope. Tissue samples were extracted and the radioimmunoassays carried out as described previously.

Results: During our studies of the regional distributions of prodynorphin-related peptides, we observed that the concentrations of α- and β-neo-endorphin were considerably higher than the concentrations of dynorphin A (plus dynorphin -A(1-8) or dynorphin B. This was especially striking in the substantia nigra and posterior pituitary and suggested to us that dynorphin A and/or dynorphin B might be converted into smaller peptide species, including LE. The fact that the levels of LE in the substantia nigra and posterior pituitary are more than twice as high as the levels of ME-Arg-Gly-Leu, a peptide derived exclusively from proenkephalin, also suggested that the LE in these structures may not be derived

exclusively from proenkephalin precursor. Leu-enkephalin and ME-Arg-Gly-Leu are present in equimolar amounts in proenkephalin and one may therefore expect their concentrations to be similar.

To determine the source of LE in the substantia nigra we made unilateral knife cuts that separate the globus pallidus from the caudate-putamen. Ten days after placement of the lesion there was a substantial decrease in α-neo-endorphin, dynorphin B and LE in the ipsilateral substantia nigra without alteration of the level of ME-Arg-Gly-Leu (Table 1).

Table 1
Effects of globus pallidus deafferentation on peptide levels in substantia nigra.

Peptide	Treatment		Substantia nigra
α-neo-endorphin	GP	Intact	7,195.5±953.5 (8)
	Deaf	Lesioned	1,053.3±353.0*(8)
			-85%
Dynorphin-B	GP	Intact	2,133.7±397.6 (8)
	Deaf	Lesioned	291.9±89.5* (8)
			-86%
Leu-enkephalin	GP	Intact	784.6±87.6 (7)
	Deaf	Lesioned	211.1±52.9*(8)
			-73%
Met-enkephalin-Arg-Gly-Leu	GP	Intact	334.9±40.7 (8)
	Deaf	Lesioned	385.1±44.2 (8)

Values are expressed as fmol/peptide/mg protein. Data are means ± SEM. Lesioned side is compared with intact sidy by paired t-test. *$p<0.001$.
Numbers in parentheses indicate the number of separate animals studied.

The most parsimonus explanation for the parallel decreases in α-neo-endorphin, dynorphin B and LE is that in the striatonigral pathway LE is made by dynorphinergic neurons. Further support for this came from an experiment in which we exposed adult male rats to intermittent foot shock of very low intensity (0.1 mA) for 20 min. Significant increases in the levels of both dynorphin B (21%) and LE (28%) were observed in the substantia nigra whereas ME-Arg-Gly-Leu concentrations did not change.

The most likely explanation for the excess of LE over ME in the posterior pituitary is that LE in the neural lobe also comes mainly from prodynorphin precursor. This idea also gained support from an experiment in which animals were under conditions of enhanced secretory activity from the posterior pituitary (e.g. 2% NaCl as a sole drinking fluid). Dynorphin-related peptides and LE were markedly decreased in the posterior pituitary following salt-loading. The magnitude of the depletion of LE was similar to that of dynorphin related peptides (Table 2). However, there was no change in the concentration of ME in posterior pituitary following salt loading.

Table 2
Peptide concentration (fmole/mg protein) in rat posterior pituitary following 100 hrs. of 2% salt-loading.

Peptide	Control	2% NaCl
ME	5,744.5 ± 425.3 (22)	5,714.2 ± 714.4 (8)
LE	12,127.0 ± 720.9 (19)	2,194.1 ± 213.8*** (9) (-81.9%)
α-Neo	24,106.0 ± 1,350.0 (22)	7,920.8 ± 816.2*** (10) (-67.1%)
β-Neo	22,378.0 ± 1,122.6 (22)	4,654.0 ± 512.6*** (10) (-79.6%)
Dyn A (1-17)	5,648.2 ± 361.3 (18)	1,390.6 ± 270.0*** (10) (-75.4%)
Dyn A (1-8)	14,375.0 ± 977.1 (19)	2,699.5 ± 315.0*** (10) (-81.2%)
Dyn B	24,548.8 ± 1,840.2 (19)	3,467.5 ± 524.4*** (10) (-85.9%)

Values are means ± SEM. *** $p<0.001$ compared to the appropriate control group (paired t-test). The number of animals is indicated in parentheses. Abbreviations: ME, Met-enkephalin; LE, Leu-enkephalin; Dyn, dynorphin: Neo, neo-endorphin.

Significance: Prodynorphin-derived peptides are potent Kappa opiate receptor agonists, while LE is a delta opiate receptor agonist. Thus the same precursor (prodynorphin) may yield ligands for different opiate receptors subtypes that can exert different actions.

Proposed Course of Study: Physiological responses of Leu-enkephalin and dynorphin will be measured electrophysiologically in substantia nigra in order to see the significance of the processing of dynorphin to Leu-enkephalin.

Publications:

1. Zamir, N., Palkovits, M., and Brownstein, M.J., Distribution of immunoreactive dynorphin in the central nervous system of the rat, Brain Res., 280, 81-97 (1983).

2. Palkovits, M., Brownstein, M.J., and Zamir, N., Immunoreactive dynorphin and α-neo-endorphin in rat hypothalamoneurohypophyseal system, Brain Res., 278, 258-261 (1983).

3. Quirion, R., Finkel, M., Mendelson, F.A.O., and Zamir, N., Localization of opiate binding sites in kidney and adrenal gland of the rat, Life Sci., 33, 299-302 (1983).

4. Zamir, N., Weber, E., Palkovitx, M., and Brownstein, M.J., Distribution of immunoreactive dynorphin B in discrete areas of the rat brain and spinal cord, Brain Res., 300 (1), 121-128 (1984).

5. Palkovits, M., Brownstein, M.J., and Zamir, N., On the origin of immunoreactive dynorphin A and α-neo-endorphin in the substantia nigra, Neuropeptides (in press).

6. Zamir, N., Weber, E., Palkovits, M., and Brownstein, M.J., Differential processing of prodynorphin and proenkephalin in specific regions of the rat brain, Proc. Natl. Acad. Sci. USA (in press).

7. Zamir, N., Palkovits, M., and Brownstein, M.J., Distribution of immunoreactive α-neo-endorphin in the central nervous system of the rat, J. Neurosci 4 (5), 1240-1247 (1984).

8. Zamir, N., Palkovits, M., and Brownstein, M.J., Distribution of immunoreactive β-neo-endorphin in discrete nuclei of the rat brain; Comparison with α-neo-endorphin. J. Neurosci 4 (5), 1248-1252 (1984).

9. Zamir, N., Palkovits, M., Weber, E., Mezey, E., and Brownstein, M.J., A dynorphinergic pathway of leu-enkephalin production in rat substantia nigra, Nature 307, 643-645 (1984).

10. Zamir, N., Palkovits, M., and Brownstein, M.J., Distribution of immunoreactive dynorphin-A (1-8) in discrete nuclei of the rat brain; Comparison with dynorphin A, Brain Res., (in press).

11. Zamir, N., Palkovits, M., and Brownstein, M.J., Distribution of immunoreactive Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu in the central nervous system of the rat, Brain Res. (in press).

12. Zamir, N., Bannon, M.J., Brownstein, M.J. and Quirion, R., Dynorphinergic pathways of Leu-enkephalin production in rat brain. Neuropeptides (in press).

13. Hoffman, D.W. and Zamir, N., Localization and quantitation of enkephalin and dynorphin in the rat hippocampus, Neuropeptides (in press).

14. Quirion, R., Martel, J.-C., St. Pierre, S.P., Gaudreau, P. and Zamir, N., Chronic haloperidol treatment differentially modulates proenkephalin versus prodynorphin-derived peptides in the basal ganglia, Neuropeptides (in press).

15. Weber, E., Sonders, M., Zamir, N., Barchas, J.D., and Evans, C.J., Distribution of metorphamide in the central nervous system, Neuropeptides (in press).

16. Zamir, N., Skofitsch, G., Bannon, M.J., Burns, S., Helke, C., Kopin, I., and Jacobowitz, D., Primate model of Parkinson's disease: Alterations in multiple opioid systems in the basal ganglia, Brain Research (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01982-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Thymocyte sodium transport activity in spontaneously hypertensive rats.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	T. Forrester	Visiting Fellow	HE, NHLBI
Others:	E. Marks	Guest Worker	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Male spontaneously hypertensive rats and Wistar-Kyoto controls age matched at 4,6, 8 and 12 weeks were sacrificed and their thymocytes isolated. The sodium content and sodium efflux rate constants of the thymocytes were determined. Cell sodium was higher in SHRs. In both groups, total and ouabain sensitive efflux fell with increasing age. However, SHRs had lower values at each age studied for total, ouabain insensitive, and furosemide/insensitive efflux. Blood pressure and total peripheral resistance were higher in SHRs. These findings may be important in the development of hypertension in the SHR.

Project Description:

Objectives: Abnormalities of sodium transport and of intracellular sodium have been described before in spontaneously hypertensive rats. These abnormalities have been said to be the cause of the hypertension. However, no attempt has been made to examine simultaneously changes in cell sodium homeostasis and haemodynamics in these animals.

Methods: Rat thymocytes were isolated from age-matched male SHR and WKY rats at 4, 6, 8 and 12 weeks of age. Thymocytes were incubated in balanced salt solution until equilibrium was regained. Then samples were taken for the measurement of intracellular sodium and potassium content. The remaining cells were loaded with ^{22}Na and the efflux rate constants were determined in buffer, as well as in the presence of ouabain, and ouabain plus furosemide. Cardiac output was measured using thermal dilution techniques.

Major Findings: At each age, cell sodium was higher in SHRs. Cell potassium was unchanged.

Table I: Thymocyte Na^+ Content (mMol/KgDS)

Age	4	6	8	12
SHR	54.5 ± .9**	53.6 ± 2.3**	58.1 ± 2.5*	44 ± 1.7**
WKY	37.9 ± 1.5	18.8 ± .7	50.4 ± 1.4	27.7 ± 0.9

* P<.005
** P<.0001

The rate constants for total and ouabain insensitive sodium efflux fell with time in both SHR and WKY rats. However, at each age, the rate constants for total, ouabain insensitive, and furosemide insensitive were lower in SHRs.

Table II:

		T	OI	FI	OS	FS
(38)SHR	4	11.72±.45**	2.5±.17**	1.65±.21*	9.35±.41	0.78±.14*
(29)WKY		14.11±.37	3.9±.11	2.42±.21	10.08±.34	1.52±.22
(43)SHR	6	10.92±.21**	2.48±.17	1.44±.19*	8.41±.15**	1.04±.31
(18)WKY		12.79±.21	2.13±.15	2.11±.14	10.66±.18	0.71±.20
(13)SHR	8	9.73±.05*	2.38±.13*	1.31±.11*	7.32±.02	0.88±.12
(15)WKY		10.19±.14	2.72±.11	1.75±.11	7.62±.02	0.93±.14
(18)SHR	12	9.00±.27**	1.95±.13*	1.32±.18*	7.33±.23	0.74±.18
(27)WKY		10.42±.16	2.78±.15	1.81±.4	7.04±.20	0.99±.16

*P<.005, **P<.001

T - total; OI - ouabain insensitive; FI - furosemide insensitive;
OS - ouabain sensitive; FS - furosemide sensitive ouabain

Blood pressure and total peripheral resistance were higher at 4 weeks of age in SHR's. Thereafter there was a steady elevation of both variables in SHR but they remained stable in WKY rats.

Significance: The fact that rate constants for total and ouabain sensitive sodium efflux fell with age in both SHR and WKY rats makes it unlikely that this finding is important in the pathogenesis of hypertension. However, the depression of the total rate constant (due to a fall in the ouabain insensitive portion) in SHR thymocytes might be responsible for the higher cell sodium content. This has pathogenetic importance for hypertension.

Proposed Course of Study: In order to elucidate the relationship between raised thymocyte sodium and the development of hypertension in SHR, rats will be divided into two groups, one of which will be subdivided and treated with antihypertensive agents. The agents chosen will include drugs known to reduce blood pressure and cell sodium and others which lower blood pressure without affecting cell sodium (e.g. (1) thiazides and (2) beta blockers). In this way we hope to dissociate the effects of cell sodium lowering and reduction of blood pressure, and describe the pathogenetic importance of all sodium in this model of hypertension.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01983-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Sodium transport in thymocytes of DOCA salt rats.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Forrester	Visiting Fellow	HE, NHLBI
Others:	E. Marks	Guest Worker	HE, NHLBI
	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Uninephrectomized rats were treated appropriately to create DOCA-salt hypertension and water drinking, saline drinking or DOC injected controls. Thymocytes were used to measure sodium transport activity in buffer and in serum. DOC salt hypertensive rats possessed higher blood pressure than controls and greater values of total and ouabain insensitive rate constants for sodium efflux. Serum caused an elevation of all components of sodium transport in both hypertensive and control rats but did so to a greater extent in the hypertensives. Serum from DOCA salt hypertensive rats contains a pump stimulant rather than an inhibitor.

Objectives: DOCA-salt hypertension is the epitome of salt-induced, volume-overload hypertension. However, there are conflicting reports concerning the presence of a circulating sodium pump inhibitor in this model and also disagreement as to whether sodium transport is enhanced or inhibited in cells free of serum. We therefore compared the haemodynamic status and cell sodium homeostasis in DOCA-salt hypertensive rats and in uninephrectomized rats receiving only DOCA, saline or water.

Methods: Male Sprague-Dawley rats were uninephrectomized and treated with the appropriate regimen to make them hypertensive or a specific control. Haemodynamics were measured with thermal dilution cardiac output techniques. Thymocytes were isolated and sodium transport and cell sodium content were determined with the cells in either buffer or serum by methods reported in Project #1 (Thymocyte sodium transport activity in spontaneously hypertensive rats.).

Major Findings: Blood pressure and peripheral vascular resistance were higher in DOCA-salt hypertension than in 1-K rats drinking water. When thymocytes from DOCA-salt hypertensive rats were incubated in buffer they had higher rate constants for total and ouabain insensitive sodium efflux than the controls. When the cells were incubated in serum all components of sodium transport in both hypertensive and control rats were increased. However, the change was greater in DOCA salt rats for total, ouabain insensitive, furosemide insensitive and ouabain sensitive constants.

CONTROL I-K H_2O

	T	OI	FI	OS	FS	N
I-K H_2O	11.87±.13	2.75±.5*	1.55±.8	8.84±.37	1.41±.19	19 Buffer
DOCA-Salt	12.25±.32	3.39±.25	1.69±.23	8.87±.36	1.89±.04	17
I-K H_2O	15.82±.19***	5.82±.18**	5.02±.17*	9.93±.22*	1.08±.26	16 Serum
DOCA-Salt	18.68±.16	6.85±.27	5.71±.25	11.85±.81	1.20±.41	12

*P<.05, **P<.005, ***P<.0005

These findings suggest that intrinsic sodium transport activity of thymocytes from DOCA salt hypertensive rats differs from that in I-K H_2O controls mainly in the ouabain insensitive component. This increase in ouabain insensitive efflux seems to be due to marginal increases in both furosemide sensitive and insensitive fluxes (neither flux alone being significantly different).

The addition of serum to thymocytes from both groups of rats caused elevation of all aspects of sodium transport measured except for the furosemide sensitive, the cotransport. The elevation in total sodium transport activity was due primarily to elevation of ouabain sensitive and the leak (ouabain furosemide insensitive efflux). Thus both passive efflux and active pumping were elevated

by serum. The surprising finding was a greater elevation of both leak and pump in the thymocytes from DOCA-salt rats incubated in serum. This finding suggests that the net effect of fresh serum on cell sodium transport in DOCA hypertension is pump stimulation rather than pump suppression.

Proposed Course of Study: Evaluation of the other controls treated with salt, and DOCA only is currently being done as well as measurement of intracellular sodium.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01984-01 HE

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Thymocyte sodium transport in Dahl rats.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)

PI:	T. Forrester	Visiting Fellow	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI

COOPERATING UNITS (if any):

LAB/BRANCH: Hypertension-Endocrine Branch

SECTION: Experimental Therapeutics

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dahl salt-sensitive and salt-resistant rats were fed either a low salt (0.3% NaCl) control diet or a high salt (8% NaCl) diet to produce hypotension in the sensitive group. Sodium transport was measured by sodium efflux method in thymocytes in either buffer or serum. Intracellular electrolytes were also measured. Results to date indicate no differences in the transport activities or intracellular sodium between sensitive and resistant groups on the low salt diet. On the high salt diet the sensitive rats had a higher cell sodium content. The effects of high salt on transport rates are being assessed.

5'4'

Project Description:

Objectives: It has been reported that there are differences in cotransport activity of red cells between Dahl sensitive and resistant rats. In view of the possibility that this sodium-loaded, volume-expanded model might possess both genetic and induced abnormalities of cell sodium homeostasis we examined both Dahl sensitive and resistant rats before and after development of hypertension.

Methods: The methods are similar to those used and reported in Project #1 (Thymocyte sodium transport activity in spontaneously hypertensive rats.). The only differences were: a) rats were sacrificed after 4 weeks on either low-salt or high-salt diets b) rate constants for sodium efflux were determined both in buffer and in serum from the same rats.

Major Findings: Dahl sensitive rats on high-salt diet (8%NaCl) displayed a higher intracellular sodium than resistant rats. Thymocyte potassium was lower in Dahl sensitive cells.

	Na^+ mMol/KgDS	K^+ mMol/KgDS
DAHL R	31.4±0.97**	559±9**
DAHL S	70.7±2.7	412±5

mMol/Kg dry solids
**P<.0001

The rate constants for sodium efflux, total ouabain insensitive, ouabain sensitive and furosemide sensitive were the same in SHR rats while on low-salt (0.3% NaCl) diet.

LOW SALT DIET (0.3% NaCl)

	T	OI	FI	OS	FS	
DAHL R (10)	12.13±.40	2.83±.22	1.42±.22	9.30±.28	1.41±.22	Buffer
DAHL S (10)	12.28±.12	2.71±.11	1.55±.19	9.57±.16	1.19±.17	
DAHL R (10)	19.91±.83	7.83±.39	6.73±.20	10.06±1.00	1.23±.28	Serum
DAHL S (10)	17.12±.69	7.41±.25	5.72±.37	9.97±.65	1.83±.39	

Incubation of thymocytes in serum caused total sodium transport activity to rise. This elevation was primarily due to the ouabain/furosemide insensitive efflux, but the ouabain sensitive and furosemide sensitive constants remained unchanged. Thus incubation in serum (as in the DOCA experiments) produced an elevation of the passive efflux of sodium.

Proposed Course of Study: The data for sodium transport in hypertensive rats are being compiled at this time along with cell electrolytes on a low-salt diet.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01985-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Treatment of essential hypertension with ketanserin, a serotonin antagonist.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.D. Levinson	Senior Staff Fellow	HE, NHLBI
Others:	H.R. Keiser	Deputy Chief	HE, NHLBI
	D.S. Goldstein	Senior Investigator	HE, NHLBI
	J. Folio	Clin. Nurse Tech.	OD, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent evidence indicates that serotonin-mediated vasoconstriction may play a role in the maintenance of essential hypertension. This study is designed to assess the antihypertensive and hormonal-metabolic effects of ketanserin, a recently-developed S_2-serotonin-receptor antagonist.

Project Description:

Objectives: Central and peripheral serotonergic mechanisms may play a role in the development or maintenance of essential hypertension in man. A newly-developed serotonin-receptor antagonist has shown a significant antihypertensive effect in both man and animals. We proposed to evaluate the effectiveness of ketanserin, a novel serotonin-2-(S_2-) receptor antagonist, as an antihypertensive agent. The effect of the drug on endocrine and cardiac variables was also assessed.

Methods: Asymptomatic hypertensive patients between 18 and 65 were studied. The trial was a double-blind, placebo-drug crossover design, with the two study periods six weeks in duration. The maximum dose of ketanserin was 40 mg twice daily. In addition to routine blood and urine studies, plasma was obtained for pituitary hormone levels, total and HDL-cholesterol, triglycerides, PRA, aldosterone, catecholamines, and plasma ketanserin levels. Cardiac output and total peripheral resistance were measured by impedance cardiography.

Results: Seven patients met all requirements for the study and are either presently participating in, or have completed the study. Except for a complaint of mild fatigue in one subject no adverse effects have been noted. Data will be uncoded and analyzed pending completion of the study.

Significance: This study will provide information concerning the safety, antihypertensive efficacy, and circulatory and hormonal effects of a novel, recently-developed antihypertensive agent. Ketanserin may also be a valuable tool for assessing the role of serotonin in blood pressure regulation.

Proposed Course of Study: To continue until a minimum of 10 subjects are enrolled.

Publications: None

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

The research in this section is primarily directed toward understanding regulatory mechanisms of biogenic amine and neuropeptide neurotransmitter systems. This includes both pre- and post-synaptic events. We are also interested in how these systems relate to blood pressure control and various disease states.

I. Biogenic Amine Systems.

a. Tyrosine hydroxylase. This enzyme is the initial and rate controlling enzyme in catecholamine biosynthesis. Prior work in our laboratory had shown that the activity of the enzyme was regulated by a protein phosphorylation reaction and that under physiologic conditions only the phosphorylated form of the enzyme is active. Since the regulation revolves around changes in the relative affinity of the electron donor, tetrahydrobiopterin (BH_4) for tyrosine hydroxylase, our recent work has been directed at this compound and its interaction with the enzyme.

We have studied the effects of tetrahydrobiopterin on the stability of pure tyrosine hydroxylase. Although in general substrates usually enhance the stability of enzymes, it was found that tyrosine hydroxylase was quickly inactivated when incubated in the presence of BH_4 and oxygen, but in the absence of the cosubstrate tyrosine. This inactivation process is time and temperature dependent. The rate with various BH_4 analogues in general varies with the effectiveness of the analog as an electron donor for the reaction; one exception to this is 6-methyl-5-deaza-tetrahydropterin which exhibits no cofactor activity but is effective in catalyzing the inactivation. Neither oxygen nor tyrosine alone or together affect the enzyme's stability in the absence of BH_4. Enzymes or compounds which scavenge various reduced oxygen species do not protect the enzyme. From this work we conclude that the reduced pterins in the absence of amino acid substrate reduce the enzyme and that the reduced enzyme reacts with oxygen to form an unstable complex which either reacts with an amino acid substrate or proceeds to an inactive enzyme. These studies have provided useful information on the reaction mechanism of this important enzyme.

Studies have also continued on the regulation of BH_4 levels. This compound is synthesized from guanosine triphosphate by a series of enzymic steps, the first of which is the conversion to dihydroneopterin triphosphate. The subsequent steps in the conversion of this pterin compound to BH_4 are not well established. Recent results from our laboratory as well as others are consistent with the idea that the second step in the pathway is the formation of 6-diketopropyl tetrahydropterin. The two keto groups are subsequently reduced to the dihydroxypropyl compound by successive reductions. The important point, however, is the likelihood that the pterin ring structure is maintained at the tetrahydro- stage throughout this portion of the pathway.

We have also examined PC-12 cells in culture during dexamethasone induction of tyrosine hydroxylase. Of particular interest is our observation that the synthesis of BH_4 is not coordinately regulated with tyrosine hydroxylase. Under

control conditions there appears to be approximately equimolar concentrations of the enzyme and BH_4. After treatment of these cells with dexamethasone, however, there appears to be 3 times as many tyrosine hydroxylase molecules as there are BH_4 molecules in the cell.

b. Neurotransmitter release. Factors regulating neurotransmitter release are as important to our understanding of synaptic function as are those related to neurotransmitter synthesis. Two systems have been used as investigational tools. 1) Release of preloaded norepinephrine from adrenergic terminals in rat heart slices and 2) release of serotonin in central synapses as measured by behavioral parameters in rats.

Work on the rat heart slice system continued and, as suggested in prior years, evidence grows for an outward transport mechanism which releases norepinephrine from nerve terminals in the heart. It has been shown that exogenous ATP inhibits this outward active transport and that this inhibition is dependent upon the energy yielding hydrolytic eleavage of ATP. Lithium, which is known to inhibit the vesicular pump for storage of norepinephrine, blocks this inhibition by ATP. This observation was considered to be evidence that the vesicle membrane system had become integrated with the plasma membrane of the neuron forming a reversible pumping system for release and reuptake of a neurotransmitter. Electron microscopic evidence has also been obtained for such a fusion of vesicular and plasma membrane.

The second release system examined used the well known behavioral syndrome known resulting from serotonin release into synapses in the CNS. Using this "5-HT syndrome," it was found that parachloroamphetamine, which causes release of serotonin, can induce an intense 5-HT syndrome in rats pretreated with reserpine. Since reserpine prevents storage of serotonin in vesicles, it is concluded that the source of the released transmitter must have been the small cytosolic pool. A number of pharmacological studies have been performed, supporting the concept that serotonin can be functionally released in a non-exocytotic manner. These included the use of monoamine oxidase inhibitors and precursors for serotonin. In both noradrenergic and serotonergic systems, the evidence suggests at least a portion of the functionally significant neurotransmitter release occurs by other than an exocytotic mechanism.

c. Phenylethanolamine N methyltransferase (PNMT). The final step in epinephrine biosynthesis is an N-methylation of norepinephrine. After development of a technique for purification of PNMT by affinity chromatography on S-adenosylhomocysteine agarose, the pure enzyme has been used for two types of studies. PNMT has been shown to be a glycoprotein. The enzyme resolved on polyacrylamide electrophoresis gives a strong reaction with periodic acid Schiff reagent. The enzyme also binds tightly to Con A and lentil lectin Sepharose, but not to wheat germ or Helix lectin. This would suggest that the sugar component may be rich in glucose or mannose, with relatively few N-acetyl sugars. In an attempt to further understand the reaction mechanism, the enzyme has been exposed to ultraviolet light in the presence of the substrate ^3H-S-adenosylmethionine. This induces a time dependent labelling of the enzyme which is blocked by S-adenosylhomocysteine. It would appear that U.V. induced reaction of the substrate with the enzyme will provide a useful probe for exploring the active site of this enzyme.

II. Regulation of Neuropeptides.

a. Substance P. In prior years we have described the interaction of both the dopamine and serotonin systems in brain with substance P neurons. Currently, attempts were made to correlate substance P binding sites with changes in substance P levels during late gestation and early development in the rat. Increases in substance P binding sites appeared to parallel the development of substance P levels in the brains of fetuses and new-born rat. Adult rat brain had a reduced level of receptors relative to the substance P content.

Angiotensin II (A II) has long been thought to be primarily involved in cardiovascular control although other functions are possible. During the past year, studies have been undertaken to characterize and compare the regulatory properties the angiotensin converting enzyme (ACE) from lung and testis. These tissues are particularly rich in this enzyme which is responsible for the synthesis of A II. Pure ACE was prepared from each of these tissues. The pulmonary enzyme was found to have a substantially larger molecular weight than the testicular isozyme, although limited proteolysis indicated some homology between these two enzymes. The enzyme from testes had a greater thermal lability, suggesting that it may be degraded more rapidly in vivo. Further examination of the testicular enzyme indicated that it was rapidly (2 to 3 weeks) lost from the tissue following hypophysectomy. Once lost, the enzyme activity could not be restored by hormone manipulation. However, continuous treatment of hypophysectomized rat with either FSH/LH or testosterone prevented the disappearance of the enzyme. Fractionation of testicular cells by elutriation indicated ACE was not present in either the Sertoli or Leydic cells, but was associated with a germinal cell type. Although the role for ACE in the testis is not known, the presence of large amounts of hormonally regulated enzyme suggest it may be involved in the processing of a peptide other than angiotensin II.

III. Receptor Mechanism.

a. Tachykinin Receptors. A series of related peptides exist in nature that have been called the tachykinins. The best studied of these tachykinins in mammals is substance P. A second related peptide, substance K, derived from the same precursor molecule has recently been identified. We have attempted to characterize the binding sites (receptors) for the tachykinins in mammalian tissue, based on relative binding affinities of the various radiolabelled peptide ligands. Previously, two types of receptors had been suggested, one that was relatively specific for substance P (SP-P) and one that preferred eledoisin (SP-E). Following the preparation of radiolabelled substance K, tachykinin receptors were examined in several tissues. It was found that an apparently previously undescribed type of receptor existed with substance K being the most potent ligand. This suggests that there may be a third type of tachykinin receptor which we have tentatively designated as SP-K.

b. Dopamine and cocaine receptors. This receptor system has been under study in our laboratory for the last several years. In previous work, it has been found that the sensitivity of this receptor on cells in the striatum can be modified in vitro by exposure to agonists and that this modification is accompanied by a specific alteration in protein phosphorylation.

During the past year, work has focused on another aspect of this striatal dopamine system. It has been shown that cocaine has a sodium-dependent specific binding site in the striatum and that this binding site is most likely located on the presynaptic dopamine terminals. It was observed that an endogenous compound can prevent binding of cocaine to this binding site. This compound has now been isolated and partially characterized. It appears to be small protein with a molecular weight of about 17,500. It has an isolectric point of 11.4 and as expected, the amino acid composition has a high proportion of basic amino acid residues. Since it is known that cocaine interferes with the dopamine reuptake system, it is postulated that the protein may interact with another regulatory protein at the dopamine recognition site.

c. Phosphatidyl inositol. Recent studies in other laboratories have suggested that the turnover of phosphatidyl inositol is linked to agonist activation of the Ca^{++} channel. Thus, when cell are preloaded with tritiated inositol, the release of phosphorylated derivatives of inositol are another index of agonist activation of a receptor. This appears to be true for synaptic function and smooth muscle contraction. During the past year, we have established this technique in our laboratory for the studies with PC-12 cells and smooth muscle cells in culture, vida infra.

IV. Post-Translational Modification of Proteins.

Our work in this area was largely confined to the carboxylmethylation of proteins. Although the physiologic role for such modification remains unknown, the relatively large amounts protein-O-carboxylmethyltransferase (PCM) and its acceptor proteins in brain suggest a possible synaptic function. PCM was purified to homogeneity from bovine brain, rat brain and human erythrocytes and the physical properties compared. Antisera to the bovine brain enzyme was generated in rabbits and immunoglobulin fractions partially purified. These antibodies were used for localization and cross-reactivity studies. With the use of a peroxidase-coupled immunocytochemical procedure, the enzyme was localized in 100 µM sections of rat brain. PCM was localized in neurons throughout the rat brain. The hippocampus was prominently labelled, with pyramidal and granule cells in all regions showing immunoreactivity. The cortex also showed extensive immunoreactivity, and neurons in all layers (I-VI) appeared labelled. The striatum and thalamus also had substantial immunoreactive PCM present, while areas of the brainstem and cerebellum were only sparsely labelled.

Western immunoblot analysis indicated that antisera generated against bovine brain PCM could label both human erythrocyte and rat brain forms of the enzyme. All forms of the enzyme exhibit similar kinetic constants, amino acid compositions, and mobility on SDS-polyacrylamide gels. Thus, there is a strong likelihood that PCM is conserved between species.

Of additional interest was the finding that methyl acceptor protein concentrations seemed to parallel the regional localization of PCM. These findings provide additional support for a role for protein methylation in neuronal function.

Initial reports suggested that calmodulin was a good substrate for PCM and that methylation of this calcium-binding protein interferes with its ability to activate phosphodiesterase (PDE). Our experiments, however, showed that PDE was

also a substrate and that methylation of PDE prevented the stimulation of this enzyme by a Ca^{++}-calmodulin, suggesting a possible role for protein methylation in the metabolism of cyclic nucleotides. Since PDE binds to calmodulin-Sepharose, the proteins that elute from such a column were examined for methyl acceptor protein activity. These calmodulin binding proteins were found to have enriched methyl acceptor activity. We therefore examined other calmodulin activated enzymes as substrates for PCM. Carboxylmethylation of various brain sources of Ca^{+2}-calmodulin-dependent protein kinases decreased calmodulin-stimulated phosphorylation. This inhibition was noted in several cytosolic and membrane preparations of Ca^{+2}-calmodulin kinase, and was also seen in purified preparations. Both tubulin and autophosphorylation patterns were inhibited in the purified enzyme. Analysis of carboxylmethylation revealed that the specific activity of methyl acceptor proteins increased from cytosol through partially purified preparations, with purified Ca^{+2}-calmodulin kinase exhibiting the highest activity. Stoichiometry indicated that approximately 1.12 mol CH_3/mol holoenzyme was incorporated in the purified enzyme. Also, acidic gel electrophoresis revealed that a protein of 62,000 daltons, which was common to all kinase prepartions, was carboxylmethylated.

Calcineurin, a phosphoprotein phosphatase is a calmodulin binding protein and is activated by calmodulin. Examination of highly purified calcineurin as a substrate for PCM showed that it could be methylated (up to 2 CH_3/mole) and as a result was no longer stimulated by calmodulin. The experiments to date therefore indicate protein carboxylmethylation may have a role in regulating calmodulin activated enzymes by methylation of these enzymes themselves rather than methylation of calmodulin.

V. Vascular Smooth Muscle Cells.

Smooth muscle cells were isolated and cultured from the media of the aorta of normotensive and spontaneously-hypertensive rats. After several passages, these cells were examined for responsiveness to specific agonists.

The cells were found to be very responsive to β stimulation. Isoproterenol stimulation resulted in an approximately 600-fold increase in cAMP levels within the cells. There were no apparent differences between cells isolated from either normotensive or hypertensive animals. However, upon the generation of large amounts of intracellular cAMP, the cells underwent dramatic morphological changes. Also of interest was the observation that these cells had minimal responses (cAMP level) to α agonists

Another approach to this problem is to measure changes in intracellular free calcium in response to agonists. To do this, the quin II technique of measuring intracellular free calcium was adapted for use in these smooth muscle cells. The smooth muscle cells loaded with quin II responded to angiotensin II with a rapid increase in apparent intracellular free calcium. Other agonists such as norepinephrine or isoproterenol did not cause an apparent increase in free calcium levels within the cell. Serotonin caused smaller but longer-lasting increment in intracellular free calcium. Arginine vasopressin was also able to cause a rapid increase in calcium content. Stimulation with either α or β agonists was ineffective in increasing the calcium content of the cells. Once again, there appears to be no fundamental differences between either normotensive or hypertensive cells.

In order to further explore the uptake or distributive change in calcium, we set up the procedure for measuring phosphatidyl inositol turnover. Studies on the uptake of inositol into cellular phospholipids was shown to require Mn^{++}. The Mn^{++}-stimulated uptake appeared to be lower in cells derived from the genetically-hypertensive rats than from control rats. However, this possible difference must be further explored. Of particular interest, however, was the finding that when cells were loaded with inositol in a serum-free medium, and exposed to an agonist that would cause a calcium influx, a rapid release of phosphoinositol was observed.

VI. Genetically Hypertensive Rats and Blood Pressure Control.

The putative role of central serotonergic neurons in the control of blood pressure in rats continued as a major project in our laboratory. Previously it was established by pharmacological and electrophysiologic studies that serotonin containing neurons in the dorsal, medial, and brainstem raphe nuclei all participated in central pressor mechanisms. In the current year, we examined the effect on the blood pressure of manipulating serotonin synthesis by precursor loading. While spontaneously-hypertensive rats generally respond to L-tryptophan loads with a decrease in blood pressure, normotensive animals have a small pressor response. By using a variety of biochemical and behavioral indices, we were able to establish several important points. 1) The blood pressure response to tryptophan reported by several laboratories is due to peripheral metabolism of this amino acid and not to a central effect. 2) Enhancement of serotonin synthesis by precursor treatment does not result in an enhance release of functionally active serotonin. 3) Serotonin that is released from central neurons appears to be derived from a cytosolic rather than a vesicular pool.

In another approach to understanding the role of central serotonin in blood pressure control, the retrograde transport of fluorescent dyes was studied. Injection of either Propidium Iodide or True Blue into the nucleus tractus solitarius (NTS) resulted in significant accumulation of dye in serotonin containing cells in the dorsal raphe area. This was established by use of a fluorescent double labeling technique. Since the NTS is a known center for cardiovascular control, it is possible that part of the serotonin pressor pathway is mediated through this center. This conclusion is strengthened by our prior observation that direct injection of serotonin into the NTS results in a marked pressor response.

Another area of research related to the spontaneously hypertensive rats relates to a possible role for nutritional factors in the expression of the sequellae of severe hypertensive. Prior work had shown that protein supplementation of rat diets somewhat low in protein was effective in reducing the incidence of cerebral lesions in stroke-prone spontaneously-hypertensive rats. While one possibility for this protective effect is enhancement of the trophic effect of sympathetic nerves on the vasculature, another might be the effect of a constituent amino acid. Since there were earlier indications that methionine might be involved, we have embarked on investigation of the effect of methionine feeding on certain membrane functions, particularly $Na\text{-}K^{+}$ ATPase and calcium uptake. These studies are still in progress, but it appears that methionine supplementation did reduce blood pressure and the incidence of cerebral lesions in rats so treated.

VII. Clinical Collaborations.

Our findings in several of our neurochemical projects have led us to establish collaborations with clinical groups. Two areas were investigated in the past year.

Observation in a number of laboratories indicated that significant losses of both norepinephrine and serotonin in addition to dopamine occurred in the brains of patients with Parkinson's disease. Since earlier studies in our laboratory had demonstrated that measurement of dopamine ß hydroxylase in human CSF was reasonable index of central noradrenergic function, we undertook a study of this enzyme in the CSF patients with Parkinson's disease. In a study of 7 patients with 7 age-matched subjects without neurological disease, we found that the level of the enzyme was reduced by about 50% in parkinsonian patients. Although the loss is not as great as a recent report in the literature, our findings provide a rationale for the use of threo-dihydroxyphenylserine as adjunct therapy to L-DOPA.

Based on our biochemical studies, we also reasoned that the synthesis of biogenic amines in the central nervous system could possibly be stimulated by administration of tetrahydrobiopterin (THB), the hydroxylase cofactor. Such a stimulation could be potentially beneficial in neurological diseases such as parkinsonism and dystonia and in endogenous depression. Precedent for such an approach was the successful use of THB in atypical phenylketonuria THB administration to seven patients with muscular dystonia provided evidence that 4 of these individuals had objective and significant improvement in their neurological symptoms. In five carefully controlled double-blind studies with individual patients with endogenous depression, relatively minor objective improvement was seen in each patient. In no case did THB approach the effectiveness of traditional antidepressant therapy. This relative ineffectiveness may be because we failed to achieve sufficiently high concentrations of the compound in the CNS. Further clinical studies are planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01843-11 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Uptake and Release of Norepinephrine in Adrenergic Nerve Endings

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Donald F. Bogdanski Pharmacologist HE NHLBI

COOPERATING UNITS *(if any)*

Dr. Victor Ferrans, Pathology Branch, NHLBI

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

New pharmacological and morphological evidence continues to be supportive of our hypothesis that Ch + -Ca ++ -stimulated neurosecretion is mediated by the outward transport of NE in vesicles fused or attached to the plasmalemma. Various chemical compounds, which can be grouped into various classes on the basis of their biochemical effects, and are known to inhibit the Mg ++ -ATP stimulated uptake of NE by isolated synaptic vesicles, all prevent or block the ATP induced inhibition of Ch + -Ca ++ stimulated neurosecretion. These compounds include Li + and reserpine, which block the vesicle pump, dicyclohexylcarbodiimide, an inhibitor of Mg ++ -ATPase which establishs proton gradients and 2,4-dinitrophenol, which dissipates stored energy. Electronmicroscopy has shown vesicles in an apparent secretory relationship with the plasmalemma. The contents of vesicles at the end of an invagination of the plasmalemma appears to be separated from the extracellular fluid by a partition. Characteristic changes in the morphology of the dense core are apparent.

551

Objectives: The efforts of the past year have been directed towards obtaining additional pharmacological and, for the first time, morphological evidence in support of our postulated Ch^{+}-Ca^{++}-stimulated neurosecretory unit.

Methods: The methods used to induce the Ch^{+}-Ca^{++} stimulated neurosecretion of ^{3}H-NE in adrenergic nerve endings were previously described in detail and are published. Briefly, rats were injected (i.v.) with ^{3}H-NE and heart ventricle slices were prepared 18 to 24 hours afterwards. After a 90 min preincubation to re-establish homeostasis groups of slices were transferred to beakers containing 20 ml of various media for incubation. Some groups were incubated in Ch^{+}-Ca^{++}, which substitutes choline chloride for NaCl in KRB along with other minor modifications. Rapid neurosecretion of ^{3}H-amines began 60 to 80 min after the start of incubation. Secretion was inhibited after the transfer of slices to beakers containing fresh Ch^{+}-Ca^{++} and ATP 3 mM. Other groups of slices were transferred to beakers containing Ch^{+}-Ca^{++}-ATP plus various substances known to block the uptake of ^{3}H-NE by isolated synaptic vesicles.

Major Findings: Efforts during the past year have focused on attempts to prevent the inhibitory effect of ATP on Ch^{+}-Ca^{++}-stimulated neurosecretion. Generally, we have employed chemical agents which other laboratories have shown to exert an inhibitory effect upon Mg^{++}-ATP stimulated uptake by isolated vesicles. Three general classes of such compounds were employed: 1) specific inhibitors of amine transport, namely, reserpine (Euler and Lishajko) and Li^{+} (Slotkin et al.); 2) inhibitors of Mg^{++}-ATPase (in our laboratory, dicyclohexylcarbodiimide (DCCD) was employed); and 3) dissipators of stored energy such as 2,4-dinitrophenol (2,4-DNP).

Effects of Li^{+} and reserpine: Preincubated slices were transferred to beakers containing Ch^{+}-Ca^{++} and incubated 80 min, at which time the maximum rate of secretion had begun. Groups of slices were then transferred to beakers containing either Ch^{+}-Ca^{++}; Ch^{+}-Ca^{++} containing ATP, 3 mM; or Ch^{+}-Ca^{++}, ATP and LiCl, 20 or 40 mM. ATP inhibited Ch^{+}-Ca^{++} stimulated neurosecretion as expected. The effect of ATP was decreased and blocked completely by 20 and 40 mM LiCl, respectively. The effects of Li^{+} lasted approximately 20 min. When added to KRB in similar concentrations, LiCl had no effect on the spontaneous depletion of tissue ^{3}H-NE.

Reserpine also blocked the effect of ATP. However, reserpine also depleted tissue stores of ^{3}H-NE.

Effects of compounds that block Mg^{++}-ATPase or dissipate energy. The inhibitor of Mg^{++}-ATPase, DCCD, also completely blocked the inhibitory effect of ATP on Ch^{+}-Ca^{++}-stimulated neurosecretion. In heart slices DCCD was effective in a concentration of 45 µM, which can be regarded as a low concentration. DCCD also inceased the release of ^{3}H-compounds in tissues incubated in KRB, but this effect was smaller than that upon ATP inhibition of neurosecretion.

The inhibitory effect of ATP on neurosecretion was moderately blocked by 2,4-DNP, which dissipates stored energy. Unlike DCCD, however, 2,4-DNP had no effect on the rate of depletion of NE in slices incubated in KRB. Non-specific effects upon neurosecretion were also elicited by N-ethylmaleimide and chlorpromazine.

Compounds affecting Mg^{++}-ATPase are regarded as acting through proton transport establishing electrical and pH gradients in isolated vesicles incubated in sucrose media. In slices, however, chloride ion is present which should prevent ATP from establishing an electrical potential. Moreover, Mg^{++}-ATP is known to stimulate uptake by isolated vesicles in phosphate buffers, but releases NE from vesicles incubated in the presence of Cl^-. Choline$^+$-Ca^{++} stimulated slices, however, appear to take up NE in the presence of ATP and Cl^-. It may be that the vesicle membrane, while in a functional relationship with the plasma membrane in an environment of normal intracellular and extracellular electrolytes, exhibits a response to ATP which differs from that of the isolated vesicle in sucrose. The determination of the properties of fused or attached vesicle membranes has always been one of our objectives. We have never fully accepted the results of experiments with isolated adrenergic vesicles in particular. With an amine T/2 of 5 to 10 min, isolated storage vesicles cannot be said to store amine. In Ch^+-Ca^{++} stimulated slices, however, vesicles at the plasmalemma may function normally in their natural environment and may react naturally under certain extracellular conditions.

Effects of NH_3. Ammonium chloride dissipates proton gradients and depolarizes nerve membranes. However, NH_4Cl depleted heart slices of their NE whether the slices were incubated in Ch^+-Ca^{++} or KRB. The depletion was not Ca^{++} dependent, nor inhibited by transport inhibitors. The tissue did not respond to ATP. It is thought that NH_3 releases NE from all vesicles in nerve endings incubated in either medium. Unlike reserpine in Ch^+-Ca^{++}, however, the response to NH_3 seems to predominate in both media. Thus, NH_3 does not appear to require the vesicle to react with the axolemma for secretion to occur.

Electronmicroscopy: Most of this work was done under commercial contract and some was done with the cooperation of Dr. Victor Ferrans of the Pathology Branch, NHLBI. Rat atria were used because of their more dense innervation, as compared to ventricles, although nerve endings are scarce in either tissue. For these experiments, rats were injected with ^{3}H-NE in the usual way, and their atria were removed on the following day. The atria were preincubated for 90 min in KRB, then transferred to media for incubation. Three groups were incubated as follows: 1) KRB, 2) Ch^+-Ca^{++}, and 3) Ch^+-Ca^{++} containing 30 μm cocaine per liter. Ten specimens of atria were immersed overnight in buffered gluteraldehyde provided by the contractor after treatment as follows: 1) The heart and blood vessels of a freshly killed rat were flushed free of blood with KRB before immersion. 2-4) slices were removed from the KRB incubation media at the end of the preincubation and after 60, 100 and 140 min of incubation. 5-7) slices were removed for Ch^+-Ca^{++} after 60, 100 and 140 min incubation. 8-10 slices were removed from Ch^+-Ca^{++} plus cocaine after 60, 100 and 140 min incubation. Liquid scintillation counting of all media showed that tissue ^{3}H-NE was retained in KRB as expected, secreted in Ch^+-Ca^{++} as expected, and secretion was blocked by cocaine as expected. Electronmicrographs of all specimens have been returned. The most important preliminary results for our purposes are that intact nerve endings were present after 140 min incubation in Ch^+-Ca^{++}. Nerve endings were better preserved, morphologically, than myocytes. Nerve endings embedded in matrices of Schwann cells were seen in cross section showing intact plasma membranes of both cell types. In other micrographs, nerve endings with well-formed mitochondria, microtubules and electron-lucent and dense-cored vesicles were seen. Several examples of dense-cored vesicles in an apparent secretory

relationship with the plasma membrane were photographed. These vesicles were located at the end of an invagination of the plasma membrane, and the contents of the vesicle were apparently separated from the extracellular fluid by a membrane. One such vesicle was apparently swollen as a result of osmotic activity. Other swollen vesicles were apparently not in contact with the axolemma. All had dense cores. Those in contact with the axolemma contained dense cores with an altered morphology. Rather than having the usual circular configuration, these dense cores were rectangular and perhaps striated with irregular borders, as though being acted upon by unknown forces.

Other morphological findings of interest were that no unequivocal examples of coated vesicles or invaginations were found in nerve endings containing dense-cored vesicles. Coated vesicles were observed in other nerve endings. Since the preponderence of photographed endings were adrenergic, the absence of coated vesicles would appear to be a significant aspect of adrenergic nerve morphology.

A possible example of true exocytosis was observed, but, of course, photographs cannot reveal the directionality of a process. Moreover, no vesicle could be positively identified at the terminus of the invagination.

Generally, the electronmicrographs are supportive of our neurosecretory model which until these studies, was based on pharmacological evidence.

Significance to Biomedical Research and Institute Programs: In our view we are accumulating important evidence which will aid our understanding of the process of secretion of small molecules, specifically neurosecretion. At this point we briefly recapitulate salient findings which have led us to formulate our hypothesis. Choline^{+} partially substitutes for Na^{+} in KRB medium although there is a rapid, Ca^{++}-dependent neurosecretion of ^{3}H-NE starting between 60 and 80 min after the start of incubation. Evidence suggests that neurosecretion is mediated by the outward transport of ^{3}H-NE from vesicles fused or attached to the plasmalemma. Thus, Ca^{++}-dependent neurosecretion is blocked by a Na^{+}-dependent action of cocaine and desipramine which are competitive inhibitors of uptake. Neurosecretion is also inhibited by ATP which is known to stimulate the uptake of ^{3}H-NE by isolated vesicles, but would not normally be expected to permeate the plasmalemma to act on intracellularly located vesicles. The effect of ATP is prevented by reserpine, Li^{+}, DCCD and 2,4-DNP, representing various biochemically active classes of compounds known to block Mg^{++}-ATP stimulated uptake of NE by the vesicle membrane. Earlier publications from this laboratory have also reported that Ch^{+}-Ca^{++} stimulated neurosecretion resembles electrically driven synaptic transmission in various aspects of their interactions with Mg^{++} and Na^{+}, their decreasing response to higher concentrations of Ca^{++} and effective substitution by Ba^{++}.

A problem with attempts to apply our results directly to theories of synaptic transmission is that inhibitors of transport generally do not inhibit synaptic transmission. A few examples of inhibition have been reported and interpreted in other contexts as side issues. In last years report we described some progress towards the resolution of the problem along the lines of K^{+} and/or depolarization inhibiting the effects of desipramine. This was not advanced during the past year.

Proposed Course of Project: Studies of the effects of metabolic inhibitors, transport blockers and other agents which inhibit the establishment or dissipation of proton gradients will continue. We will continue our efforts to obtain more electronmicrographic examples of, as well as pharmacological evidence of fused or attached vesicle and plasma membranes as well as to observe other significant features of the morphology of normal and stimulated nerve endings. We have found an example of fused membranes, possibly with elimination of layers. By inducing a sustained period of rapid secretion, it is thought that Ch^{+}-Ca^{++} stimulation will increase the probability of our finding more examples of fused vesicle and plasma membranes. Attempts to perform our own experiments on the pharmacologically or electrically driven release of neuronal ^{3}H-NE will be made.

Publications:

Bogdanski, D.F., The effect of exogenous adenosinetriphosphate on the choline-calcium stimulated release of ^{3}H-norepinephrine in rat heart ventricle slices. J. of Neural-Transmission 57: 213-232, 1983.

Bogdanski, D.F., Monovalent cation dependency for the inhibition of outward transport of ^{3}H-norepinephrine. Neuropharmacology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01850-15 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of the Spontaneously Hypertensive Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martina Diolulu Research Fellow HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.	0.	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The amount and quality of dietary protein has been shown in earlier studies to influence the incidence of pathological lesions associated with severe hypertension in certain rat models. Specifically low protein diets resulted in a greater number of cerebral lesions and stroke in the stroke-prone spontaneously hypertensive rat (SHR-SP). The current study is an attempt to understnad the biochemical basis for the protective effect of dietary protein. SHR-SP were maintained on natural diets containing different levels of protein, some of which were supplemented with methionine, and various aspect of membrane calcium metabolism were examined. Preliminary results suggest that sarcoplasmic reticulum isolated from heart has greater Ca++ binding capacity when animals are maintained on a high protein diet. Likewise there was an apparent slight increase in Ca++ ATPase of this organelle in animals receiving the higher protein diet. Pathological examination of the tissues also revealed fewer lesions in the rats supplemented with protein.

GPO 904-917

Objectives: A number of experimental approaches indicated a possible cell membrane defect from both hypertensive patients and experimental hypertensive animals. Such membrane defects which could serve a pathogenic role in activating the mechanism maintaining high BP include: altered ion permeability (Jones, Circ. Res. 33: 563-572, 1973; Suppl. 1, 34-35: 117-122, 1974), defective membrane pump systems (Postnov et al.; Clin. Sci. Mol. Med. 51: 169s-172s, 1976) and reduction of Ca^{2+} binding capacity of cardiac and arterial sarcoplasmic reticulum (SR) as well as erythrocyte membranes (Postnov, Pflugers Arch. 379: 191-185, 1979; Derynck et al., Hypertension 3: 191-195, 1981). Alteration of the ability of subcellular membranes such as SR to bind Ca^{2+} may result in increase of intracellular free Ca^{2+} concentration which may probably be one of the important factors for the increase in muscular tone. If associated with the vascular smooth muscle, such an abnormal intracellular distribution of Ca^{2+} would cause the increase of vascular resistance resulting in hypertension.

Studies in our laboratory and others have demonstrated that high protein diet or high fat diet containing moderate amount of protein significantly retards the development of stroke, reducing the accompaning incidence of cerebral and cardiovascular lesions, while low protein diet accelerates the disorders. In the present study we intend to investigate the mechanism by which high protein diet protects against the above mentioned disorders. Several biochemical parameters such as membrane ATPase activities and Ca^{2+}-transport activities of fractions isolated from ventricular myocardium of hypertensive rats under different diets were examined.

Methods:

Experimental animals: Spontaneously-hypertensive rats (SHR), the stroke-prone substrain (SHR-SP) and normotensive control Wistar-Kyoto rats (WKY) were obtained from the Animal Production facilities at the NIH. Rats were maintained on a 12 hour day-night cycle and received natural diets prepared to contain various protein levels. Some diets had supplementary methionine added. Blood pressures were measured at weekly intervals by a tail cuff plethesmographic technique. At the conclusion of the experiments animals were sacrificed, some for pathological and some for biochemical measurements described below.

Tissue preparation: Rats were decapitated and their hearts excised, trimmed of atria and the ventricles placed in ice-cold oxygen bubbled Krebs-Ringer solution, pH 7.4 in which EGTA was substituted for Ca. Then the ventricles were blotted dry, weighed and chopped into small pieces with scissors.

Preparation of cardiac myofibrils (Mf) and sarcoplasmic reticulum (SR): Because cardiac preparations are extremely labile resulting in poor yield of SR (Inesi et al.; Am. J. Physiol 207: 1339-1344, 1964, Kyuzo et al.; Jap. Heart J. 15: 475-484, 1974) cardiac myofibrils and microsomes enriched in SR were prepared by a modification of the methods of Fanburg et al. (J. Biol. Chem. 239: 2298-2306, 1964 and Harigaya and Schwartz, Circ. Res. 25: 781-794, 1969), respectively. Chopped ventricles were homogenized with 4-5 volumes of ice-cold medium containing 0.1 M KCl, 25 mM Tris-maleate buffer (pH 6.8) and 1 gm/200 ml of α-tocopherol using a polytron (PT-20 Brinkman Instrument Co). This lipid antioxidant protects mitochondria (Tappel Arch. Biochem. Biophys. 80: 326-332, 1959)

and microsomes (Tappel et al., Nature 185: 35, 1960) from the hematin-catalyzed peroxidation of lipid during homogenization.

The homogenate was centrifuged at 600 g for 20 min. The resulting pellet (contains Mf) and supernate (contains SR) were saved. This procedure was repeated 3 times, combining and saving the supernatants. Then the sediment was resuspended in 25 mM Tris-maleate buffer (pH 6.8) and passed through four layers of cheese cloth for removal of coarse materials. The myofibrils were washed twice by repeated suspension in buffer and centrifugation at 600 g for 20 min.

The saved supernatant which contains SR was also passed through cheese cloth and sedimented at 10,000 g for 30 min. The pellet was discarded and the supernate centrifuged at 37,000 for 50 min to sediment the crude SR. This pellet was resuspended in 10 ml of 0.6 M KCl and extracted for 30 min in the cold to render the contaminating actomyosin soluble (Uchidi et al., Biochim. Biophys. Acta 104: 287-289, 1965; Martonosi, J. Biol. Chem. 243: 71-81, 1968). The suspension was again sedimented at 37,000 g for 50 min. Finally, both the Mf and SR were suspended in Tris-maleate buffer containing 40% sucrose and stored at -70°C until assayed.

Protein assay was determined by the Bradford method.

Cardiac myofibrillar and SR ATPase activities were determined by monitoring liberated inorganic phosphate according to the method of Fiske Subbarow. Reaction mixture for ATPase activity of Mf was: 25 mM Tris-maleate buffer pH 7.2, 5 mM $MgCl_2$, 5 mM NaN_3 in the presence of 1 mM Ca^{2+} (total ATPase) or 1 mM EGTA (Basic ATPase) and that of cardiac SR was: 25 mM Tris-maleate pH 6.8, 0.12 M KCl, 0.3 mM $MgCl_2$ and 2.5 mM ATP in the presence of 0.5 mM Ca^{2+} or 1 mM EGTA. Ca^{2+} activated ATPase activity was defined as the difference between the basic and the total activity.

Measurement of cardiac SR Ca^{2+} transport activity: Calcium binding by reticular vesicles was measured using ^{45}Ca in a Millipore filter method. The reaction mixture contained 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM ATP, 1.1 mg/ml membrane protein and 6.5, 13.0 and 26.0 μM $CaCl_2$ containing ^{45}Ca (0.5 μCi) in 25 mM Tris-maleate buffer, pH 6.8 in a total volume of 2 ml. The reaction was started by the addition of ATP after a 30 second to 5 min of incubation period at 37°C. After the incubation periods, the reaction was terminated by immediately filtering the sample mixture through a 0.45 Millipore filter (HA 45, Millipore Co) connected to a vacuum pump. The filter was washed trice with 2 ml of assay medium containing 5 mM EGTA. Using a liquid scintillation counter, calcium binding was estimated from the radioactivity of the filtrate and filter. The maximum Ca^{2+}-binding (number of binding sites) and binding constants were calculated from the double reciprocal plot of bound and free Ca^{2+}

Major Findings of Preliminary Studies:

1) Weekly or biweekly measurements of BP by the tail pulse pick-up method without anesthesia show that while neither diet nor salt loading (to reduce the time required for the development of stoke) had any significant effect on the BP of normotensive rats, the BP of hypertensive rats on lower protein diet was

significantly higher than that of rats on higher protein or the methionine supplemented diet.

2) Myofibrils from hearts of all test animals showed essentially the same basic and Ca^{2+}-activated ATPase activity. However, when compared to rats on lower protein diet, the maximum Ca^{2+}-binding capacity and binding constant of cardiac SR were higher in animals fed with a higher protein diet. Also, the SR from rats on higher protein diet had significantly elevated Ca^{2+}-activated ATPase activity.

3) Quantitative estimation of calmodulin in whole brain show that rats on a high protein diet had greater amount of calmodulin than rats on lower protein diet.

4) Histological study showed significant microscopic lesions in the kidneys, brains and hearts of test animals on lower protein diet but fewer lesions in those rats on high protein diet.

5) Red blood cells of untreated stroke-prone rats have higher levels of Na^{+} than do the cells of normotensive WKYs.

Proposed Course of Project: With emphasis on stroke-prone spontaneously hypertensive rats (SHRSP), we intend to investigate the effects of dietary protein on:

1) Vascular permeability; a activity of membrane bound enzymes (ATPases), b Ca^{2+}-uptake and c ion fluxes across cell membranes of rat hearts, kidney and brains.

2) Protein content and/or synthesis in vascular walls.

3) Because calmodulin is a ubiquitous Ca^{2+} binding protein, we plan to characterize calmodulin effects on Ca^{2+} transport and (Ca^{2+}-Mg^{2+})-activated ATPase activity in erythrocytes and cardiac SR isolated from our test animals.

4) Phospholamban is an intermediate protein and an SR component strongly associated with the Ca^{2+}-pump. In the presence of ATP, ionized Ca^{2+} and Mg^{2+} the phosphorylation of phospholamban preceeds the activation of Ca^{2+}-Mg^{2+}-ATPase and consequently the stimulation of Ca^{2+}-uptake. Using SR preparations from our test animals, we plan to determine phosphoprotein formation by the method of Katz and Blostein, Biochem. Biophys. Acta 389: 314-325, 1975.

Publications:

1. Yamori, Y., Horie, R., Tanase, H., Fujiwara, K., Nara, Y. and Lovenberg, W.: Possible role of nutritional factors and the incidence of cerebral lesions in stroke-prone spontaneously hypertensive rats. Hypertension 6: 49-53, 1984.

2. Kobayashi, K., Tarazi, R.C., Lovenberg, W. and Rakusan, K.: Coronary blood flow in genetic cardiac hypertrophy. American Journal of Cardiology 51: 1744, 1983.

3. Lovenberg, W. and Yamori, Y.: Nutritional factors and cardiovascular disease. Clin. Exp. Hypertension A6 (1&2): 417-426, 1984.

4. Lovenberg, W.: Possible relationship between nutrition and cardiovascular disease. In Yamori, Y. and Lovenberg, W. (Eds.): Nutritional Prevention of Cardiovascular Diseases, 1984, in press.

5. Lovenberg, W. and Yamori, Y.: Dietary Protein, the central nervous system and hypertension. Annals of Internal Medicine, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01859-13 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dopamine-Beta-Hydroxylase As A Biochemical Marker (New Title)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Jeffrey H. Hurst Guest Worker HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dopamine-beta-hydroxylase (EC 1.14.17.1; DBH) is the biosynthetic enzyme which catalyzes the conversion of dopamine to norepinephrine in sympathoadrenal cells and CNS neurons. During the exocytotic release of norepinephrine, DBH protein is released along with the neurotransmitter. This released DBH protein can be measured in both the circulatory pool and the cerebrospinal fluid. We have assayed CSF DBH levels in non-medicated Parkinson's disease patients, and found a significant decrease in their DBH levels to 41% control. This finding suggests that alterations and/or deficits central noradrenergic function are occurring in Parkinson's disease.

Objectives: The primary objective was to observe if alterations in CSF DBH activity occurred in Parkinson's Disease, Alzheimer's disease and other neurological conditions. As DBH levels in the CSF may accurately reflect central noradrenergic functioning, changes in CSF DBH levels could reflect altered activity or neuronal degeneraton in noradrenergic systems.

Rationale of Study: A report by Nagatsu and co-workers (BioMed Res. 3: 95-98, 1982) indicated that Parkinson's disease patients had CSF DBH levels reduced to only 6% of control values. However, their Parkinson's disease patients had CSF taken from the lateral ventricle (during stereotaxic surgery), while control patients had normal lumbar punctures for CSF sampling. Thus, we were interested in determining if the difference in sampling site could account for the noted decrease in CSF DBH levels, or if Parkinson's disease is indeed associated with deficits in CSF DBH levels.

Methods: All human CSF samples were obtained by lumbar puncture on patients with Parkinson's disease (as assessed by clinical symptoms) and normal volunteers at the inpatient unit, NINCDS. CSF samples were frozen immediately on dry ice, and the 15th ml sample was used for assessing DBH enzyme activity so as to obviate any CSF gradient effect. Samples were assayed within 24 hr of sampling (90% of samples assayed within 3 hr), as deterioration of CSF DBH activity occurs within prolonged storage at -80°C (Lerner et al., Biol. Psychiatry 13: 685-694, 1978). DBH activity was measured using a radiochemical assay procedure, and all samples were assayed in triplicate.

Monkey CSF samples were obtained from ventricular and lumbar sites, and were handled and assayed as for human samples.

Major Findings: CSF samples from ventricular and lumbar sites in monkeys showed no significant difference in DBH levels, suggesting that sampling site for CSF is not critical for DBH determination.

Eight control patients showed a mean CSF DBH level of 0.540 ± 0.09 units/ml. In contrast, eight Parkinson's Disease patients showed CSF DBH levels of 0.223 ± 0.034 units/ml, a reduction that was statistically significant ($p<0.01$). There was no significant correlation between CSF DBH levels and age in either group, nor was there any correlation between CSF DBH levels and severity of parkinsonian symptoms as assessed by the five stage clinical rating scale (Hoehn and Yahr, Neurology, 17: 427-442, 1967).

Significance to Biomedical Research and Institute Programs: The symptoms of disease are believed to reflect deficits in central dopaminergic functioning. Our results indicate that significant decrements in CNS noradrenergic function are also a component of the disease. Such changes in noradrenergic function could account for certain cognitive deficits observed in Parkinson's disease.

Publications: Hurst, J.H., LeWitt, P.A., Burns, R.S., Foster, N.L., and Lovenberg, W.: CSF dopamine-beta-hydroxylase activity in Parkinson's disease. Neurology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01874-07 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthesis of Epinephrine

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Jeffrey H. Hurst Guest Worker HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Phenylethanolamine N-methyltransferase (EC 2.1.1.28; PNMT) catalyzes the conversion of norepinephrine to epinephrine in the adrenal medulla and brainstem. Utilizing milligram quantities of pure bovine adrenal PNMT obtained by our purification scheme, we have studied the biophysical characteristics of the methyltransferase enzyme. Studies on the photolabelling of PNMT with 3H-[methyl]-S-adenosyl-L-methionine (AdoMet) indicate the relatively rapid formation (10 min) of a covalent bond between AdoMet and PNMT under ultraviolet conditions. This labelling can be blocked by the presence of PNMT substrate, phenylethanolamine, but was unaffected by free radical scavengers. Additional work has revealed that PNMT is a glycoprotein, with preliminary findings of mannose- and/or glucose-rich sugar chain(s).

Objectives: PNMT is the final biosynthetic enzyme in the synthesis of the neurotransmitter/neurohormone, epinephrine. A better understanding of its biophysical properties may give us better insight into its physiological regulation, as well as determining whether PNMT and other small molecule AdoMet-dependent methyltransferases may be considered a family of enzymes as regards biophysical characteristics.

Methods: Bovine adrenal PNMT was purified according to our protocol (J.H. Hurst et al., Biochem. Biophys Res. Comm. 112: 1061-1068, 1983). PNMT was incubated with ^{3}H-(methyl)-AdoMet for various times under UV light at 4^{o}C. Attempts to modify the photolabelling reaction were made by adding substrate (phenylethanolamine), free radical scavengers (dithiothreitol), and AdoMet antagonists (singefungin). Binding was assessed by 10% SDS-PAGE and by fluorography. Further, the site on the PNMT enzyme that was photolabelled was compared to that labelled on protein-O-carboxylmethyltransferase (E.C. 2.1.1.24; PCM), a large molecule (protein) methyltransferase, by analyzing peptide maps made from Staphlococcus V8 protease digests of labelled enzymes.

Assessment of whether PNMT contain any sugar residues was initially carried out using periodic acid-Schiff stain. Further work assessed the ability of PNMT to bind to immobilized lectins of differing affinities. Lectins utilized included Concanavalin A, lentil lectin, wheat germ lectin, and Helix pomatia lectin.

Major Findings: ^{3}H-[methyl]-AdoMet is able to radiolabel PNMT under UV light conditions in a time-dependent manner. The addition of free radical scavengers to the reaction did not block the binding. Exposing either PNMT alone or AdoMet alone to UV light, followed by addition of the second component, resulted in no binding, suggesting that any reactive intermediate which is formed is short-lived, and/or that both enzyme and AdoMet must be in close apposition during the formation of a binding intermediate in order to form a covalent, UV-induced bond.

Proteolytic mapping of PNMT and PCM after photolabelling indicated markedly different patterns, suggesting that the two enzymes have very different primary structures. Also, the fragments that were labelled with ^{3}H were different in PCM and PNMT.

PNMT showed a positive staining pattern using periodic acid-Schiff stain. Preliminary findings using affinity chromatography columns composed of immobilized lectins indicated that PNMT avidly binds to Con A and lentil lectin, suggesting that the protein is rich in glucose or mannose residues. The absence of binding to wheat germ or Helix lectin suggested either a lack of N-acetyl-glucosamine and N-acetyl-galactosamine residues, or that any such residues are structurally constrained from entering into binding reactions with the appropriate lectin. Further work is in progress to delineate the glycoprotein nature of PNMT.

Significance of Research: An understanding of the biophysical properties of PNMT should lead to a better understanding of its regulation in vivo, and thus to a better perspective in in vivo formation of epinephrine. It is, of course, still unclear what role epinephrine may play, either centrally or peripherally,

in the etiology of hypertension. Nevertheless, there is increasing evidence that central epinephrine systems may play a role in blood pressure regulation.

Publications:

Hurst, J.H., Billingsley, M.L., and Lovenberg, W.: Photoaffinity labelling of methyltransferase enzymes with S-adenosylmethionine: effects of methyl acceptor substrates. Biochem. Biophys. Res. Comm., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01879-07 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Dopamine Receptors Regulation during Sub- and Supersensitivity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Others:	Melvin Billingsley	Staff Fellow	HE NHLBI
	Michael Jennewein	Guest Worker	HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

~~Hypertension-Endocrine~~

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism for the desensitization of striatal dopamine receptors appear to be heterologous. Prolonged incubation of striatal slices with D-1 receptor agonists result in a decreased responsiveness of adenylate cyclase to further stimulation by dopamine, NaF or cholera toxin. Experimental evidence indicates that the functional efficiency of the G/F protein is reduced. Prolonged incubation of striatal slices with D-1 receptor agonist is also associated with an increased phosphorylation of membrane proteins. Preincubation of striatal slices with haloperidol greatly reduces this increase, while sulpiride fails to alter the increase in phosphorylation.

Objectives: In recent years the biochemical processes underlying supersensitivity or desensitization of neurotransmitter receptors have obtained considerable interest. These receptors are viewed to be entities composed of various molecular components with distinct functions. Alterations in receptor properties can be elicited by prolonged occupancy or deprivation of recognition sites by or from specific agonists. Therefore, our investigative interest focuses on the role of the various molecular components of the dopamine receptor as a possible site of alteration specific for the functional state of this receptor.

Methods: Rat striatal slices in Krebs-bicarbonate buffer pH 7.4 at constant oxygenation were incubated in the presence of various dopamine receptor agonists or (and) antagonists for a prolonged period of time. At the end of the incubation, various components of the dopamine receptor complex were studied including: responsiveness of adenylate cyclase to dopamine; changes in G/F protein activity were studied indirectly by measuring the stimulation of adenylate cyclase by NaF and cholera toxin or directly by photoaffinity labeling of the G/F protein with 8'-azido-GTP and phosphorylation of striatal membrane proteins.

Major Findings: Prolonged exposure of striatal slices to D-1 receptor agonists attenuates the subsequent stimulation of adenylate cyclase by dopamine or other D-1 receptor agonists. This subsensitivity of adenylate cyclase to stimulation by dopamine is associated with a decreased affinity of recognition sites for dopamine, a reduced coupling efficiency of G/F-protein, a decrease in the content of membrane-bound calmodulin and an increased phosphorylation of membrane proteins. The degree of phosphorylation is dependent on agonist concentration and duration of incubation. Preincubation of striatal slices with haloperidol prevents the desensitization of D-1 receptor-linked adenylate cyclase and greatly reduces the increase in phosphorylation elicited by prolonged incubation with dopamine. In contrast, preincubation of striatal slices with sulpiride, a specific D-2 receptor antagonist, failed to prevent any of biochemical alteration associated with desensitization of dopamine receptor. Prolonged incubation of striatal slices with LY 141865, a specific D-2 receptor agonist, elicited an increase in phosphorylation in only one of the protein band that were phosphorylated after prolonged incubation with D-1 receptor agonists. These results suggest that an increased phosphorylation of certain membrane proteins may be associated with the desensitization of D-1 receptor-linked adenylate cyclase.

Significance to Biomedical Research and Institute Programs: The goal of this research project is to improve the present understanding of the function of post-synaptic neurotransmitter receptors. The outcome of these studies will render information on the action of transmitters or cotransmitters and will improve our understanding of altered receptor function in various cardiovascular illnesses.

Publications:

1. Memo, M. and Hanbauer, I.: Phosphorylation of membrane proteins in response to persistent stimulation of adenylate cyclase-linked dopamine receptors in slices of striatum. Neuropharmacology 23: 449-455, 1984.

2. Hanbauer, I., Memo, M. and Billingsley, M.: Role of calmodulin in the regulation of dopamine receptor function. In P. Greengard, et al (Eds.): Adv. in Cyclic Nucleotide and Protein Phosphorylation Research, Vol. 17. Raven Press, New York, 1984, pp. 521-527.

3. Hellstrom, S., Hanbauer, I., Commissiong, J., Karoum, F. and Koslow, S.: Role and regulation of catecholamines in carotid body. In Hanin, I (Ed.): Dynamics of Neurotransmitter Function, Raven Press, New York, 1984, pp. 31-38.

SCF

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03505-06 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Pteridine Cofactors on the Stability of Tyrosine Hydroxylase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)

PI:	Donald M. Kuhn	Pharmacologist	HE NHLBI
Others:	Eleanor Bruckwick	Chemist	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tyrosine hydroxylase purified to homogeneity from cultured rat pheochromocytoma cells is inactivated by incubation with its reduced pterin cofactors L-erythro- tetrahydrobiopterin (BH4), 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin (6MPH4) and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH4). Each of the two diastereoisomers of L-erythro-tetrahydrobiopterin inactivates tyrosine hydroxylase but the natural (6R) form is much more potent than the unnatural (6S) form at equimolar concentrations. The pterin analog 6-methyl-5-deaza-tetrahydropterin, which has no cofactor activity with tyrosine hydroxylase, also inactivates the enzyme whereas the oxidized pterins 7,8 dihydrobiopterin and biopterin do not. The inactivation process is both temperature and time dependent and results in a reduction of the V_{max} for both tetrahydrobiopterin and tyrosine. Neither tyrosine nor oxygen inactivates tyrosine hydroxylase. However, incubation of the enzyme with BH4 in the absence of oxygen prevents pteridine-induced inactivation. The phosphorylated form of tyrosine hydroxylase is also quite sensitive to inactivation by reduced pteridines.

SL 9

Objectives: Tyrosine hydroxylase is the initial and rate limiting enzyme in the synthesis of the catecholamines. Purification and characterization of this important enzyme has been hindered as a result of its instability. Since it is a general phenomenon that many enzymes are more stable in vitro in the presence than in the absence of their substrates, we investigated whether the pteridine cofactors (cosubstrates) would alter the stability of native tyrosine hydroxylase as well as that of tyrosine hydroxylase which had been phosphorylated by cAMP-dependent protein kinase. Phosphorylated tyrosine hydroxylase is known to be much more labile than the native enzyme form.

Methods: Tyrosine hydroxylase was assayed by the method of Lerner et al. (Neurochem. Res. 3: 641, 1978). For stability studies, enzyme was incubated at 37°C in room air for 0-60 min in the presence or absence of BH_4, $6MPH_4$, or $DMPH_4$ or other substances. Aliquots were removed at 15 min intervals for the enzyme assay.

For phosphorylation experiments, tyrosine hydroxylase was incubated with 0.5 mM ATP, 5 mM Mg^{2+}, and 20 µg of purified catalytic subunit of cAMP-dependent protein kinase. After 10 min at 37°C the enzyme was mixed with various concentrations of a pteridine cofactor and incubation was continued for up to 20 min at which time the enzyme was assayed for activity.

Tyrosine hydroxylase was purified to homogeneity from cultured rat pheochromocytoma cells. Cells were sonicated into 50 mM potassium phosphate buffer containing 20% (w/v) sucrose and 0.1 mM EDTA. Following centrifugation at 100,000 x g, the tyrosine hydroxylase in the supernatant was treated with ammonium sulfate (30-42%) and chromatographed sequentially on Sepharose CL-6B, DEAE, phenyl-Sepharose, and Fractogel HW55(P). The final enzyme appeared pure on SDS-polyacrylamide gel electrophoresis.

Major Findings: Tyrosine hydroxylase is quite stable throughout the entire preincubation period. After 60 min preincubation at 37°C there is not more than a 10% loss of catalytic activity. The addition of BH_4, 6 MPH_4, or $DMPH_4$ surprisingly caused a rapid loss of tyrosine hydroxylase activity. The inhibitory effects of the pteridines were caused by reduced forms since neither biopterin nor dihydrobiopterin caused a loss of activity. The two diastereoisomers of BH_4 (referred to as 6R- and 6S-BH_4) were also tested for their effects on the hydroxylase and it was found that the inhibitory potency resided almost completely in the natural 6R-BH_4 form. The pterin analog 5-deazapterin and the pyrimidine 2,5,6 triaminopyrimidinone, both of which have little, if any, cofactor activity with tyrosine hydroxylase, both produced inactivation of the enzyme. Since both of these compounds bind to tyrosine hydroxylase, the pterins appear to inactivate the hydroxylase via a direct effect. Various antioxidants and radical scavengers were tested for their influence on the pterin inactivation of tyrosine hydroxylase. It was found that catalase, superoxide dismutase, peroxidase, dithiothreitol, ferrous ions, and ascorbic acid could not protect tyrosine hydroxylase from pterin inactivation. Finally, neither oxygen nor tyrosine altered tyrosine hydroxylase after preincubation.

If tyrosine hydroxylase was phosphorylated prior to exposure to BH_4, the inactivation by BH_4 was more rapid and occurred at very low BH_4 concentrations (5-20 µM). The inactivation of the phosphorylated form of tyrosine hydroxylase

was oxygen dependent since inactivation did not occur in the presence of nitrogen. The loss of activity in the phosphorylated form appears to be irreversible and does not reflect a change in activity of the protein kinase which phosphorylates the hydroxylase.

Significance to Biomedical Research and Institute Program: These experiments represent the first demonstration that tyrosine hydroxylase undergoes significant ligand effects. The inactivation of tyrosine hydroxylase resided in the natural, reduced forms of the pterin cofactors and appears to be a direct effect of the molecule on the enzyme structure. Tyrosine hydroxylase is activated *in vivo* by phosphorylation and the phosphorylated form or the enzyme is quite labile. The present studies suggest that BH_4 may modulate the stability of tyrosine hydroxylase under a situation (phosphorylation) where the affinity of the enzyme for its cofactor is increased. This mechanism may represent another means by which tyrosine hydroxylase is regulated. Furthermore, the ability of BH_4 to inactivate phosphorylated tyrosine hydroxylase may reflect the action of BH_4 as a hydrophobic probe more than that of a cofactor.

Proposed Course of Project

1. Pheochromocytoma cells in culture will be incubated with various concentrations of BH_4 and the penetration of BH_4 into the cells and its effects on tyrosine hydroxylase therein will be investigated.

2. The ability of various phospholipids such as phosphatidylserine and phosphatidylinositol to mimic the effects of the reduced pteridines on the phosphorylated form of tyrosine hydroxylase will also be investigated since these agents can activate tyrosine hydroxylase under the appropriate conditions.

Publications:

Kuhn, D.M. and Lovenberg, W.: Serotonin and metabolites. In: Methods in Biogenic Amine Research, I. Najatsu and T. Nagatsu (eds.), 1983, pp. 515-548.

Kuhn, D.M. and Lovenberg, W.: Tyrosine hydroxylase: Inactivation by reduced pterin cofactors. Biochem. Biophys. Res. Comm. 117: 894-900, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03511-05 HE

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of the Brain Serotonergic in Blood Pressure Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William A. Wolf	Guest Worker	HE NHLBI
Others:	Donald M. Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of the serotonergic neuronal system on blood pressure of normotensive WKY rats and spontaneously hypertensive rats (SHR) was studied by injecting L-tryptophan, the metabolic precursor to brain serotonin. It was observed that the dose- and temporal effects of tryptophan on blood pressure were not related to the effects of tryptophan on serotonin levels throughtout the brain. The dextro-rotary isomer of tryptophan was no different from L-tryptophan in its ability to increase brain serotonin but D-tryptophan does not alter blood pressure in SHR or WKY. Taken together, these results indicate that the cardiovascular effects of L-tryptophan are not mediated by brain serotonin.

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Objectives: The role of the brain serotonin system in blood pressure regulation is not well understood. This is due in part to the difficulty in specifically interacting with the central serotonin system by pharmacological means. To avert this problem, investigators often inject L-tryptophan, the precursor amino acid for serotonin synthesis. Presumably, tryptophan is converted to serotonin only within those cells containing the required enzymes (tryptophan hydroxylase and aromatic L-amino acid decarboxylase). The objective of this study was to assess the effects of L-tryptophan on blood pressure of normotensive and hypertensive rats and to correlate changes in serotonin neurochemistry to changes in blood pressure.

Methods: Male Wistar-Kyoto and spontaneously hypertensive rats were housed in temperature and humidity controlled isolator chambers. Blood pressure was measured by a tail cuff method which uses a photoelectric sensor to detect tail pulses. The method is used at ambient temperature unlike all other indirect tail cuff methods where it is required to heat animals in order to dilate tail vessels.

Major Findings: Injections of L-tryptophan increased blood pressure in the WKY. Doses of 25, 50, and 100 mg/kg each increased blood pressure by 10-15 mm Hg. This effect was observed within 30 min of tryptophan injections and blood pressure returned to normal by 4 hours. On the other hand, SHRs showed little response to 25 and 50 mg/kg of L-tryptophan but responded with substantial reductions (7-30 mm Hg) in blood pressure at 30, 60, and 120 mm after injection of 100 mg/kg. Neurochemical analysis indicated that brain serotonin levels were increased uniformly by approximately 1.2-1.4 fold while brain tryptophan levels increased over 10 fold at the highest dose. The dose- and temporal effects of L-tryptophan on blood pressure were not related to the effects of L-tryptophan on serotonin neurochemistry. For example, 100 mg/kg of L-tryptophan increased brain serotonin no more than doses of 25 or 50 mg/kg but this dose increased blood pressure in WKY and decreased blood pressure in SHR. Furthermore, injections of equimolar doses of D-tryptophan increased brain serotonin and 5-hydroxindoleacetic acid to the same extent as L-tryptophan in the SHR, but D-tryptophan does not alter blood pressure.

Significance to Biomedical Research and Institute Program: These experiments indicate that L-tryptophan does have a significant antihypertensive effect in hypertensive animals. Although tryptophan is frequently used to manipulate brain serotonin levels (serotonin itself does not cross the blood brain barrier), it does not appear that this amino acid is a useful probe of the brain serotonin system. These studies are leading us to re-evaluate a number of physiological processes judged to be dependent on brain serotonin by virtue of their responsiveness to tryptophan injections.

Proposed Course of Project:

1. Investigate the effects of 5-hydroxytryptophan on blood pressure in SHR and WKY. This amino acid is another precursor of serotonin which is frequently used to alter brain serotonin.

2. Investigate the effects of non-indole metabolites of tryptophan on blood pressure. These include quinolinate, kynurenine, kynuramine, and indolepropionic acid.

Publications:

Howe, P.R.C., Kuhn, D.M., Minson, J., Stead, B. and Chalmers, J.P.: Evidence for a bulbospinal serotonergic pressor pathway in the rat brain. Brain Research 270: 29-36, 1983.

Kuhn, D.M. and Lovenberg, W.: Tryptophan hydroxylase. In: Chemistry and Biochemistry of Pterins, Blakely, R.L. and Benkovic, S. (Eds.), Wiley, New York, 1983, in press.

Wolf, W.A. and Kuhn, D.M.: Antihypertensive effects of L-tryptophan are not mediated by brain serotonin. Brain Research 295: 356-359, 1984.

Wolf, W.A. and Kuhn, D.M.: Effects of L-tryptophan on blood pressure in normotensive and hypertensive rats. J. Pharmacol. Exp. Ther., 1984, in press.

Wolf, W.A., Lovenberg, W. and Kuhn, D.M.: Serotonin and central regulation of blood pressure. In P.M. Vanhoutte (Ed.): Serotonin and the Cardiovascular System. New York, Raven Press, 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03513-05 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Molecular components of the striatal dopamine uptake system

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Ingeborg Hanbauer	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The reuptake system for dopamine includes at least three functional components: a specific carrier system with dopamine recognition sites, a regulatory site characterized by high-affinity recognition sites for uptake inhibitors and a coupling device that links together the regulatory and carrier sites. Sodium-dependent ^{3}H-cocaine binding sites are located exclusively on dopaminergic terminals in caudate nucleus. An endogenous polypeptide present in synaptosomes displaces ^{3}H-cocaine from its binding sites in a non-competitive fashion and inhibits dopamine uptake. Its apparent molecular weight is 17,500 and its amino acid composition shows the presence of high amounts of basic amino acid residues. It is postulated that the cocaine recognition sites may be associated with the dopamine carrier through this basic regulator protein.

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Objectives: Recent findings of radioligand binding studies suggested the existence of at least two types of dopamine uptake inhibitors. One that acts directly on the dopamine recognition site (Hill coefficient = 1) and the other type that appears to act on recognition sites operative for an endogenous regulator of the dopamine uptake system (Hill coefficient < 1). This regulatory site has been studied in our laboratory using as a probe high-affinity ^{3}H-cocaine binding which in the presence of sodium exclusively labels striatal dopaminergic terminals. The present project is aimed at studying the function of this regulatory protein in the regulation of dopamine uptake. Furthermore, our interest will focus on determining whether an endogenous ligand exists with specific recognition sites on the regulatory protein that may be functional in regulating dopamine uptake.

Methods: ^{3}H-cocaine binding was measured in crude membranes prepared from caudate nucleus in the presence of sodium. The reaction was terminated by rapid filtration of the sample over Whatman GF/B filters. Filters were washed 3 times with buffer, dried, and counted to determine bound radioactivity.

The endogenous inhibitor of cocaine binding was extracted with 1 M CH_3COOH at 90°C, the supernatant fraction was chromatographed on Biogel P-10 and further purified on HPLC (μ-Bondapak C_{18}, Waters Inc.). The uptake of ^{3}H-dopamine was studied in striatal slices using Krebs bicarbonate buffer pH 7.4 saturated with 95% O_2 and 5% CO_2. Non-specific ^{3}H-dopamine uptake was determined in the presence of 10^{-4} M nomifensine. ^{3}H-dopamine was extracted from slices with 0.1 M HCOOH and the amount of radioactivity was measured by scintillation counting.

Major Findings: Sodium-dependent ^{3}H-cocaine binding was used as a marker for dopaminergic nerve terminals. In specific, the recognition sites for cocaine appear to be located on a regulatory protein operative in the ^{3}H-dopamine uptake system. ^{3}H-cocaine was displaced from its binding sites by a polypeptide that was extracted and purified from synaptosomal membranes. This protein has an apparent molecular weight of 17,500 and is basic (pI = 11.4). It non-competitively displaces sodium-dependent cocaine binding on striatal membranes because it decreases the B_{max} value without changing the $^{3}K_D$ value. In the presence of protease inhibitors, this protein inhibits also ^{3}H-dopamine uptake in striatal slices.

Proposed Course of Project: Future research will be aimed at characterizing the molecular components of the dopamine uptake system. The characterization of the endogenous regulator protein will include studies on tryptic digestion of this protein to determine whether it is a precursor of an endogenous effector including several copies of the same molecule or if it could be a protein functioning as a regulator for dopamine uptake. Since studies involving long-term treatment of rats with cocaine showed that cocaine recognition sites in striatal membranes were up-regulated, indicating that cocaine may act as an antagonist on these recognition sites, striatal tissue will be studied for the possible presence of endogenous agonist for cocaine recognition sites.

Significance to Biomedical Research and Institute Programs: The outcome of this research will improve the understanding of neurotransmitter reuptake mechanisms. Release and reuptake processes control the amount of transmitter available at their post-synaptic receptor sites and thus determine the state of

sensitivity of these receptors. These studies will render information on the action and (or) side effects of drugs and on altered receptor function during aging or various mental disorders.

Publications:

Hanbauer, I., Kennedy, L.T., Missale, M.C. and Bruckwick, E.C.: Cocaine binding sites located in striatal membranes are regulatory sites for dopaminergic synapsis. Adv. in Biochem. Psychopharmacology, Raven Press, New York (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03516-05 HE

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis, Distribution and Biological Role of Substance P and Its Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mei-Lie Swenberg	Research Chemist	HE NHLBI
Other:	Stephen Buck	PRAT Fellow	HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Six distinct fractions that exhibited binding activity were isolated from rabbit antiserum against substance P (SP) by pH gradient elution on a CM-Sephadex column chromatography. Isoelectric points (IP) and SP-binding characteristics were determined on some of these fractions. Two of them had IP of 9.0 while another one had a value of 8.0. One of the major binding fractions appeared to have binding sites with two different affinity for SP while a minor fraction with a single binding site. Partially purified antiidiotypic antibodies were prepared and shown to compete with SP for binding to SP receptors in brain and salivary gland. The IC-50 for cold SP and antiidiotype serum for these two tissues were 7.5 and 1.4 mM and 1:1320 and 1:600 dilution, respectively. In a separate study, the membrane protein from cerebral cortex was solubilized and purified with wheat germ lectin Sepharose chromatography. Affinity gel was prepared by coupling the solubilized membrane protein on Affi-Gel 10 and its binding activity and the effect of Mn^{++} were measured. This is the first report confirming that the SP binding activity of the solubilized membrane protein is intact. Finally, the relationship between the development of SP receptor and the neuropeptide itself was studied. High affinity specific binding sites for SP appeared to develop late in the gestation period and this development is largely in parallel with the development of substance P content of the tissue.

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Objectives: Substance P (SP) appears to serve a role as a neurotransmitter and neuromodulator in both the CNS and periphery. It seemed important to know more about how this neuropeptide interacts with its receptor. In order to achieve this, there are several approaches that could be pursued, these include: 1) extraction, purification and characterization of the receptor molecule, 2) derivation of new probes for studying the interaction of the peptide with its receptor, such as an antiidiotypic antibody, and 3) determination of the developmental pattern for SP and its receptor and correlation of this with changing function. These types of experimentation should lead to better understanding of the role of SP.

Methods: The receptor characteristics in membranes from various rat brain tissues at various stages of development were conducted as reported by Buck et al., Life Sci 34: 497, 1984 and Lee et al., Mol. Pharmacol. 23: 563, 1983. Commercial monoclonal antibody was purified with SP affinity column (SP coupled with Affi-Gel 10) and eluted with 5 M $MnCl_2$. Antiidiotypic antibodies were prepared by immunization of a rabbits with this purified monoclonal SP antibody.

Antisera from rabbits immunized with BSA-SP was fractionated on a CM-Sephadex column with a pH gradient elution between pH 6.8 and 8.0.

The SP receptor solubilized in a buffer containing triton X-100 or CHAPSO was purified by a wheat germ lectin-Sepharose column chromatography and elution with 0.2 N N-acetylglucosamine.

Molecular weight and homogeneity of protein preparations were determined with a PAA electrophoretic system. Thin layer isolectric focussing was used to measure the isolectric point of proteins.

Major Findings: The SP receptor protein was successfully solubilized from rat brain cortical tissue. Following elution from the lectin column, the binding of SP could be measured following the adsorption of the receptor protein to an affinity column. Examination of the receptor protein coupled to an affinity column showed that in tricine buffer Mn^{++} promoted specific binding but combination of both inhibit binding saturation of ^{3}H-SP on rat brain. Further characterization of this isolated receptor protein is planned.

An antiidiotypic antibody for SP was produced and reported in last years annual report. The apparent presence of this antibody was deduced by the ability of this serum to displace SP from the original antibody. In the current year the interaction of this antiidiotype with the SP receptor has been examined in rat brain and salivary gland. Dilution of serum to 1:1300 and 1:600 are sufficient to displace 50% SP from binding sites on membranes of rat brain and salivary gland, respectively.

In considering possible difference in functions of SP in developing and mature brain, we have examined ^{3}H-SP binding and compared the changes in binding to those of SPLI levels. We found that substantial amount of low affinity (K_D > 10 μM) ^{3}H-SP binding on G15 through G18 (gestation day) with additional appearance of high affinity (K_D = 0.5 nM) binding at G20 through G23 prior birth. High affinity binding persist through birth and into the first two weeks after

birth (≃200 fmole/mg protein). The adult high affinity ^{3}H-SP binding level was 50 fmole/mg protein. SPLI was transiently elevated at G21-22 and during P7 through P21 (postnatal day).

In the periphery, the uterus has one of the highest concentrations of SP receptor. Of interest is the observation that during pregnancy the apparent number of SP receptors in the uterus decreases.

Significance to Biomedical Research and Institute Programs: The peptide substance P appears to be an important neurotransmitter in the CNS. Previous studies in our laboratory have demonstrated the nature of the interaction of this neuronal system with the dopamine and serotonin neuronal systems. The current experiments were designed to further understand both the development of the SP system and its interaction with the endocrine system in CNS and peripheral system. The work is directed at understanding the role of SP in cardiovascular control and other physiologic systems.

Proposed Course of Project:

1) Continue the study of a possible new function of SP in prenatal development and the mechanism of its abrupt decrease at birth.

2) Attempt to isolate the SP receptor and study its regulation and function in sensory nervous system and neuroendocrine system.

3) Hybridoma cell line will be obtained to produce antibodies against isolated SP receptors of CNS and peripheric as tools for studying the function and regulatory effect of SP and its receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03520-04 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Dopamine Receptor Regulation in Schizophrenic Illness

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Other:	Gabriele Panza	Guest Scientist	HE NHLBI
	Jack Grebb	Psychiatrist	PP NIMH

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation of adenylate cyclase was studied in nucleus caudatus, nucleus accumbens, hippocampus and cerebellar cortex of control and schizophrenic brains. The adenylate cyclase activity in the dopamine-rich brain areas of schizophrenics was more enhanced by SKF 38393, NaF or GppNHp than in similar areas of control brains. In contrast, the stimulation of adenylate cyclase by Na+ and GppNHp was similar in cerebellar cortex and hippocampus of controls and schizophrenics. It is inferred that the functional efficiency of the G/F protein that facilitates adenylate cyclase activation is increased in dopamine-rich brain areas of schizophrenic subjects. These findings suggest that aberrant dopaminergic transmission in brain of schizophrenics could be due to a basic defect in the function of the G/F protein.

Objectives: A number of direct and indirect investigations indicate that the dopamine receptor could be one of the sites where functional impairment occurs in schizophrenia. Reports in the literature showed that the density of D-2 dopamine recognition sites was increased in dopamine-rich brain areas of schizophrenic subjects and dopamine receptor antagonists relieve the symptoms of schizophrenia. To obtain information on whether dopamine receptor function is altered in schizophrenic brain, various biochemical markers of pre- and post-synaptic processes linked to dopamine neurotransmission will be studied.

The post-synaptic processes concern the mediation by coupling proteins of the interaction between dopamine recognition sites and the transducer-adenylate cyclase. Long-lasting occupancy of dopamine receptors by agonists or antagonists triggers changes in pre- and postsynaptic dopaminergic mechanisms. Thus, the availability of dopamine in the synaptic cleft may determine in situ the functional state of the dopamine receptor. Release and reuptake of dopamine have to be considered as major mechanisms determining the amount of dopamine which is present in the synaptic cleft. In view of these considerations, studies on the molecular mechanism for dopamine reuptake may render important information on pathological alterations on the level of dopaminergic synapses.

Several distinct classes of calcium antagonists are important therapeutic agents in cardiovascular and numerous other medical conditions. Several of these compounds have been shown to specifically bind to brain membrane preparations. On basis of structural similarities, several neuroleptics were shown to mimic the action of a specific group of calcium channel antagonists. It will be of importance to evaluate whether the clinical action of these neuroleptics may relate to their calcium antagonist action.

Methods:

Studies on Post-synaptic Dopamine Receptor Regulation

Adenylate cyclase activity is measured as previously described (Clement-Cormier et al., Proc. Nat. Acad. Sci., USA, 71 1113-1117, 1974). Incubations are carried out in presence or in absence of various concentrations of NaF (1 mM, 2.5 mM, 5 mM, 10 mM), is Gpp(NH)p (5 uM, 10 uM, 50 uM, 100 uM) or dopamine receptor agonist. The amount of cyclic AMP formed was measured by radioimmunoassay. Since specific subunits of the G/F protein interact specifically with D-1 and D-2 dopamine receptors, the biochemical characteristics of this protein were studied in dopamine-rich brain areas from schizophrenic and non-schizophrenic subjects. Photoaffinity labeling with ^{32}P-8-azido GTP is carried out on membrane extracts prepared with 1% n-octyl glucoside followed by SDS-slab gel electrophoresis. The migration characteristics of the various subunits and their affinity for radiolabeled GTP are determined.

Studies on Dopamine Uptake

Na^+-dependent cocaine binding can be used as a marker for dopaminergic nerve terminals. Membranes are prepared from dopamine-rich brain areas of schizophrenic and non-schizophrenic subjects. From saturation isotherms the K_D and B_{max} values for the specific cocaine binding on membranes is estimated by Scatchard analysis. An endogenous regulator protein present in synaptosomes

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which was shown to modulate Na^+-dependent cocaine binding in striatal membranes and ^3H-dopamine uptake into striatal slices will be measured by radioimmunoassay using a specific antibody directed toward this protein.

Studies on Calcium Channels: The specific binding characteristics of various radiolabelled calcium antagonists to membrane preparations from specific brain areas of schizophrenic and non-schizophrenic subjects are evaluated by Scatchard analysis. Various groups of calcium antagonists and neuroleptics are examined for their ability to modify the radioligand binding. The effect of long-term treatment with either calcium antagonists or neuroleptics on the specific binding of these radioligands is evaluated in animals model experiments.

Major Findings: The coupling efficiency of the G/F protein in dopamine-rich brain areas of schizophrenic subjects is greater than in similar brain areas of non-schizophrenic subjects. This inference was derived by two types of results 1) the stimulation of adenylate cyclase by NaF, Gpp(NH)p or SKF 38393 in nucleus caudatus and nucleus accumbens showed a higher degree of activation of this enzyme in schizophrenic subjects. In contrast, stimulation of adenylate cyclase by NaF or Gpp(NH)p in cerebellar cortex or hippocampus was similar in schizophrenic and non-schizophrenic subjects. 2) Photoaffinity labelling with ^{32}P-8-azido-GTP striatal membrane extracts showed a higher incorporation of labeled GTP into various protein bands containing GTP subunits extracted from schizophrenic brains than from control brains.

Proposed Course of Action: Studies are in progress to quantitate the amount of G/F protein in different brain regions from normal and schizophrenic subjects. Studies on the stimulatory and inhibitory subunits of the G/F protein are planned to render information on possible alterations of the coupling of D-1 and D-2 type dopamine receptors in schizophrenic brains.

Significance of Biomedical Research: This research project is designed to obtain a better understanding of biochemical defects that may exist in schizophrenic illness. Better insight on basic biochemical mechanisms linked to dopaminergic transmission will be studied on the level of pre- and postsynaptic processes and will provide a basis for a more specific and effective therapy.

Publications:

Memo, M., Kleinman, J. and Hanbauer, I.: Coupling of dopamine D_1 recognition sites with adenylate cyclase in nuclei accumbens and caudatus of schizophrenics. Science 221:1304-1307, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03525-03 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biological Regulation of Angiotensin-I Converting Enzyme

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Paul Velletri Staff Fellow HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.9	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two processes have been studied that may regulate angiotensin-I converting enzyme (ACE) activity. One process involves the control of intrinsic molecular activity by endogenous inhibitors. A second process involves hormonal control of a unique ACE isozyme in testis. The endogenous inhibitor(s) is soluble, dialyzable and heat stable under conditions favoring low oxygen tension. It appears to be a sulfhydryl-containing compound as N-ethylmaleimide blocks its activity and it is readily oxidizable. The inhibitor has not yet been isolated nor have many of its molecular characteristics been described. Testis provides a good model to study hormonal control of tissue levels of ACE. In the rat, the pituitary is essential for the development and maintenance of a unique isozyme of ACE that occurs in testes. This isozyme shares a certain degree of amino acid sequence homology with the well-described pulmonary enzyme, but is about 55,000 daltons smaller. The smaller testicular isozyme is less thermally stable than its larger pulmonary counterpart and this characteristic may affect its rate of renewal by rendering it more susceptible to proteolysis. Once the testicular isozyme has been allowed to disappear following hypophysectomy (either pre-pubescently or in mature rats), it is difficult to re-initiate synthesis with gonadotropins or androgen. However, if hormone replacement is started on the first day following hypophysectomy, follicle stimulating hormone/luteinizing hormone combination or testosterone, but not human chorionic gonadotropin, can prevent the loss of testicular ACE activity. These results, in combination with studies performed on purified cell populations from the testes, suggest that testicular ACE is associated with cells of the seminiferous tubules, most probably germinal cells.

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Objectives: Investigations on the biological regulation of angiotensin-I converting enzyme (ACE; kininase II; dipeptidyl carboxypeptidase; E.C. 3.4.15.1) have been extended to the study of tissue-specific regulation of ACE in lung and testes. Objectives of the studies were to ascertain mechanisms that control the intrinsic molecular activity of ACE or the tissue levels of ACE. Our interest in the biological regulation of ACE stems from its presumed involvement in the turnover of many biologically active peptides in the periphery and central nervous system.

Our laboratory has attempted to clarify the nature of the endogenous ACE inhibitor described in the intramural project report of 1982-1983. Furthermore, we have attempted to delineate differences between the pulmonary and testicular isozyme and to determine the biological consequences of the presence of isozymes in lung and testis. We have indirectly studied the turnover of ACE in lung and testes and have attempted to describe the hormonal influences that may affect ACE turnover in testes. We have also attempted to determine the cell type with which testicular ACE is associated.

Methods: ACE activity was measured under the standard conditions of Cushman and Cheung (Biochem. Pharmacol. 20: 1637, 1971) as described in previous reports. Purification of pulmonary or testicular ACE was accomplished by using modifications of the affinity chromatographic technique originally described by El-Dorry et al. (J. Biol. Chem. 257: 14128, 1982). Briefly, the technique consisted of absorbing crude tissue detergent extracts to an epoxy-Sepharose column to which was linked the specific site-directed ACE inhibitor, N-α-[1-(S)-carboxy-3-phenylpropyl]-L-lysyl-L-proline. Elution of ACE, which was homogeneously pure as assessed by the reducing, denaturing conditions of SDS polyacrylamide gel electrophoresis, was accomplished with buffer containing inhibitor. The inhibitor was dialyzed away to achieve a catalytically active enzyme.

Studies on the endogenous ACE inhibitor employed centrifugally-prepared 100,000 x g cytosolic fractions from any number of tissues of interest. Comparisons to a variety of purified cytosolic components were also carried out. Thermal lability studies on crude and purified ACE preparations were ordinarily conducted at 55^{o}C, as the thermal lability of ACE at physiologic temperatures was too slow for practicality. Hormonal studies were conducted with Pergonal^{R} (menotropins: follicle stimulating hormone/luteinizing hormone; FSH/LH), human chorionic gonadotropin (hCG) or testosterone acetate (TEST). Further details of all these studies will be described in the following section.

Major Findings: As described in last year's project report, whole rat brain cytosol has been shown to possess a heat-stable, dialyzable endogenous inhibitor of ACE. Further experiments have been conducted over the past year to characterize the endogenous inhibitory factor(s). These studies have employed purified pulmonary ACE as a source of catalytic activity. Besides whole brain cytosol, 100,000 x g supernatant fractions prepared from liver, kidney, heart and testes have also been shown to contain comparable inhibitory activities. It was also noted that the endogenous inhibitory factor was stable to boiling (100^{o}C) for 30 min, but only under conditions that reduced oxygen tension in the supernatant. Hence, inhibitory activity of cytosol was lost if native or boiled cytosol was allowed to incubate for up to 90 min at 37^{o}C. The loss of activity could be attenuated by incubating cytosol in the presence of 0.1% ascorbic acid,

a powerful anti-oxidant that has little effect on basal ACE activity. Loss of activity could be completely prevented by conducting incubations in a nitrogen atmosphere in a Thunberg tube. Incubations carried out at $0^{\circ}C$ also prevented loss of cytosolic inhibitory activity.

Although dialyzable, the inhibitory factor did not appear to be destroyed by Pronase® incubations. It was not extractable in a stable form into 1 N acetic acid, chloroform, ethyl acetate or n-butanol. Of interest was the observation that pre-incubation with N-ethylmaleimide, a sulfhydryl blocking reagent, destroyed inhibitory activity of cytosol. These results pointed to the distinct possibility that the endogenous inhibitory factor was in fact a sulfhydryl-containing compound. Of further interest was the fact that both glutathione (GSH) and cysteine (CYS), but not oxidized glutathione (GSSG), oxidized CYS or methionine, were potent inhibitors of ACE. Furthermore, the kinetics of GSH inhibition were virtually identical to those of crude cytosol. Unfortunately, two observations mitigated against GSH or CYS being the endogenous inhibitory factors: (1) both GSH and CYS were demonstrated to be stable to heating under conditions that resulted in the loss of cytosolic inhibitory activity; chromatographed cytosol depleted of inhibitory activity did not destabilize GSH or CYS; and (2) levels of endogenous cytosolic GSH and CYS - alone or in combination - were far lower than would be required to elicit the degree of inhibitory activity seen in cytosolic preparations. Hence, although the identity of the endogenous ACE inhibitor has proved difficult to determine, certain characteristics have been described. From these studies we were able to conclude that endogenous inhibitors in certain tissues may be components of an ACE regulatory system that controls the intrinsic molecular activity of ACE.

A second form of biological regulation of ACE that has been explored in some detail and that may prove to be an intriguing model system to study the biological regulation of peptide hydrolases has been the control of testicular ACE. Two aspects of testicular ACE regulation were studied: (1) the relationship between isozyme structure and thermal stability, and how these two factors may affect in vivo turnover of ACE; and (2) the relationship between hypophyseal gonadotropins, androgens and the induction and maintenance of testicular ACE and the cell type to which the isozyme is located.

Experiments conducted with both crude and purified preparations of pulmonary and testicular ACE indicated that the pulmonary isozyme was more thermally stable (i.e., did not lose catalytic activity as rapidly) than the testicular isozyme. The pulmonary isozyme was noted to be larger by about 55,000 daltons and showed portions of homology and heterology with the primary amino acid sequence of testicular ACE. As would be predicted by the conformer equilibrium hypothesis, the less thermally stable isozyme would be expected to denature (unfold) more readily at any temperature in vivo and hence would be more available for general proteolysis. It was also noted that chelators of the essential zinc moiety of ACE greatly potentiated the thermal lability of both isozymes. These studies allowed us to conclude that the binding of zinc to ACE is not only important for catalytic activity but renders the enzyme more thermally stable. Furthermore, it appears as though the testicular isozyme is less stable than the pulmonary isozyme and therefore may be degraded more readily in vivo. As the specific enzyme activity of ACE in lung and testis is approximately the same, we

have concluded that the rate of ACE renewal must be greater in testes, as the overall susceptibiity of testicular ACE to proteolysis and degradation appears to be greater than that of the pulmonary isozyme.

The fact that testicular ACE may have a greater rate of renewal than pulmonary ACE is interesting in light of the fact that testicular (but not pulmonary) ACE appears to be hormonally regulated. Our initial studies have indicated that testicular ACE is not associated with Leydig or Sertoli cells but most probably with certain stages of germinal cells of the seminiferous tubules. We have observed that the testicular isozyme does not develop in rats that have been hypophysectomized prior to puberty. Furthermore, if mature rats that have developed the testicular isozyme are hypophysectomized, enzyme activity is completely lost within 2-3 weeks. Once ACE is lost, re-initiation of its synthesis is difficult to achieve. Hormone replacement with FSH/LH, hCG or TEST brings back only a small percentage of the expected ACE activity. However, if hormone replacement is conducted from the first day of hypophysectomy in mature rats, loss of activity can be prevented by dosing animals with FSH/LH or TEST, but not with hCG. These results confirm our observations with distinct cellular preparations of the testes, which suggest that the enzyme is associated with seminiferous tubules but not Leydig or Sertoli cells.

Significance to Biomedical Research and Institute Programs: A definition and understanding of the biological regulation of ACE is important for at least four reasons. Firstly, ACE recognizes many biologically active peptides or their precursors as substrates. Hence, changes in the intrinsic molecular activity or in the tissue level of ACE can have profound consequences on the turnover of any number of peptidergic systems. Secondly, with the clinical introduction of the ACE site-directed specific inhibitors in the treatment of essential hypertension and congestive heart failure, an understanding of what controls the de novo synthesis and proteolytic degradation of ACE may be important in determining the long-range effects of chronic treatment with drugs such as captopril and enalapril. Thirdly, ACE is one of the few peptide hydrolases with a well-defined function in at least certain tissues (such as lung). It can therefore be viewed as a model peptide hydrolase whose mechanisms of biological regulation may be pertinent to other similar metallopeptidases. Fourthly, the function of ACE in testis is as yet unknown. Nevertheless, the fact that it is found in abundance in that tissue, at specific activities comparable to what is detected in lung, is strongly suggestive of a role in the physiology of the testes. Discovering its role in this tissue may have important implications for the chronic use of active site-directed inhibitors and for our knowledge about testicular function.

Originally, our interest in ACE concentrated on its role in hypertension, a project well in keeping with the overall goals of the Hypertension-Endocrine Branch of NHLBI. However, the general biological question of how ACE activity and levels are controlled became of increasing interest to us and appeared to be of fundamental interest in understanding the functions of this particular peptide hydrolase. Because this peptide hydrolase appears to be involved in the processing of so many peptides, an understanding of how it is regulated might aid in our understanding of factors that control levels of many different biologically active peptides, some of which may directly or indirectly relate to

blood pressure control. Furthermore, understanding the hormonal control of testicular ACE, and its function in testes, may extend our knowledge of testicular biochemistry and physiology and of the many factors that relate to male fertility.

Proposed Course of Project: The following areas of research are being actively pursued:

1) The biochemical and physiological function of ACE in testes will be studied. A solution to this problem will be greatly facilitated when the cell type in testes with which ACE is associated is defined (see item #2). The function of testicular ACE will be approached both immunologically and pharmacologically with active site-directed specific inhibitors such as captopril and enalapril. Chronic ACE inhibition in testes will be related to androgen production, spermatogenesis and parameters of seminal characteristics.

2) The cell type with which ACE is associated in the testes will be defined. At present, preliminary studies are highly suggestive of a localization of testicular ACE to seminiferous tubules, but not Sertoli cells. These studies are being conducted with the collaboration of Dr. Maria Dufau and her colleagues in the NICHHD.

3) The biological regulation of synthesis and degradation of ACE in endothelial cells and the appropriate cell type of testes will be clarified. Many of these studies will employ ^{35}S-methionine and other tracer amino acids that are incorporated in substantial amounts into ACE. Specific incorporation into ACE will be determined by immunoprecipitation and by affinity-gel separation techniques that are presently being refined in our laboratory.

4) Antibodies against rat pulmonary and testicular ACE will be developed in rabbit and/or goat.

5) The relationship between the apoenzyme from lung or testes to the divalent cation present at the active site will be examined for its effects on catalytic activity.

Publications:

1. Velletri, P.A. and Lovenberg, W. Presence of an endogenous inhibitor of dipeptidyl carboxypeptidase in rat brain stem. Neurochem. Internat. 6: 265, 1984.

2. Velletri, P.A., Billingsley, M.L. and Lovenberg, W. Thermal denaturation of rat pulmonary and testicular angiotensin converting enzyme: effects of chelators and $CoCl_2$. J. Biol. Chem., 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03533-02 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Interactions between Carboxylmethylation and Phosphorylation in CNS Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Melvin L. Billingsley	Staff Fellow	HE NHLBI
Others:	Paul Velletri	Staff Fellow	HE NHLBI
	Donald Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*

Professor Robert DeLorenzo, Yale University

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Carboxylmethylation of various brain sources of Ca+2-calmodulin-dependent protein kinases decreased calmodulin-stimulated phosphorylation. This inhibition was noted in several cytosolic and membrane preparations of Ca+2-calmodulin kinase, and was also seen in purified preparations. Both tubulin and autophosphorylation patterns were inhibited in the purified enzyme. Analysis of carboxylmethylation revealed that the specific activity of methyl acceptor proteins increased from cytosol through partially purified preparations, with purified Ca+2-calmodulin kinase exhibiting the highest activity. Stoichiometry indicated that approximately 1.12 mol CH3/mol holoenzyme was incorporated in the purified enzyme. Also, acidic gel electrophoresis revealed that a protein of 62,000 daltons, which was common to all kinase preparations, was carboxylmethylated. This suggests that carboxylmethylation can modulate Ca+2-calmodulin dependent phosphorylation by directly carboxylmethylating the source of kinase activity.

S-89

Objectives: This study extends previous observations which indicated that protein-O-carboxylmethyltransferase (PCM; E.C. 2.1.1.24) could inhibit Ca^{+2}-calmodulin-stimulated phosphorylation in rat brain cytosol. Purified Ca^{+2}-calmodulin kinase and various partially purified fractions were analysed for 1) the ability to incorporate methyl groups and 2) what effect such incorporation had on Ca^{+2} calmodulin stimulated phosphorylation.

Methods: The following fractions containing Ca^{+2}-calmodulin dependent protein kinase activities were prepared and used in this study: 1) whole rat brain cytosol, 2) calmodulin-depleted cytosol using fluphenazine Sepharose, 3) calmodulin-depleted synaptic membranes, 4) homogeneous Ca^{+2}-calmodulin-dependent protein kinase (gift of Dr. R.J. DeLorenzo), and 5) cytosolic eluates from calmodulin-Sepharose. Carboxylmethylation in these fractions was quantitated using purified PCM and [^{3}H]-S-adenosylmethionine (^{3}H-AdoMet) as a methyl donor after acidic precipitation and basic hydrolysis of [^{3}H]-protein methylesters. The effects of carboxylmethylation on subsequent Ca^{+2}-calmodulin dependent phosphorylations was determined by first carboxylmethylating fractions 1-5 with purified PCM and 10 µM AdoMet for 10 min, followed by analysis of protein kinase activity, using [γ^{32}p]-ATP as a phosphate source. Phosphoproteins were resolved using sodium dodecyl sulfate gel electrophoresis, followed by autoradiography. Ca^{+2}-calmodulin was added after carboxylmethylation and the reaction was inhibited with 100-200 µM S-adenosylhomocysteine: this prevents calmodulin carboxylmethylation during the phosphorylation phase.

Major Findings: Carboxylmethylation of the source of Ca^{+2}-calmodulin protein kinase activity was found to inhibit the subsequent phosphorylation of various proteins in the cytosolic fractions. When purified Ca^{+2}-calmodulin kinase was examined, the phosphorylation of tubulin, a preferred substrate, was markedly inhibited. In addition, the subunits of the enzyme (M_r = 62,000 and 53,000) exhibited autophosphorylating activity, and this was also inhibited after carboxylmethylation. Thus, carboxylmethylating conditions were found to inhibit Ca^{+2}-calmodulin dependent phosphorylations in brain cytosol, membranes, and purified forms of the kinase.

The pattern and amount of carboxylmethylation was examined in these fractions. Cytosol had the lowest specific activity of methyl acceptor proteins; the specific activity increased with increasing purity of the enzyme. Purified Ca^{+2}-calmodulin kinase incorporated approximately 1.12 mol CH_3/mol holoenzyme (M_r = 600,000). Acidic gel electrophoresis of carboxylmethylated proteins revealed that a 62,000 dalton protein was carboxylmethylated in all preparations, including the purified kinase. This suggests that carboxylmethylation of the 62,000 dalton kinase subunit prevents Ca^{+2}-calmodulin activation of phosphorylating activity.

Significance to Biomedical Research: These findings suggest that Ca^{+2}-calmodulin dependent protein phosphorylation in brain may be regulated by carboxylmethylation. Thus, conditions which affect the overall status of carboxylmethylation (i.e., ratio of S-adenosylmethionine, S-adenosylhomocysteine) may modify the extent of phosphorylation in the brain. Drugs which specifically inhibit protein methylation may also modify the status of Ca^{+2}-calmodulin-stimulated phosphorylation.

Proposed Course of Research: This project is completed and will be terminated.

Publications:

Billingsley, M., Hanbauer, I., and Kuhn, D.: Role of calmodulin in the biochemical regulation of neuronal function. In: Handbook of Neurochemistry, Vol. 8, (ed. A. Lajtha), pp. 201-215, 1984.

Billingsley, M.L., Velletri, P.A., Lovenberg, W., Kuhn, D.M., Goldenring, J., and DeLorenzo, R.J.: Is Ca^{+2}-calmodulin dependent protein phosphorylation in rat brain modulated by carboxylmethylation? J. Neurochem. 1984.

S 7 1

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03536-02 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Carboxylmethylation of Calmodulin-Binding Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Melvin L. Billingsley	Staff Fellow	HE NHLBI
Others:	Randall Kincaid	Staff Fellow	CM NHLBI
	Paul Velletri	Staff Fellow	HE NHLBI
	Donald Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Metabolism, NHLBI

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Calmodulin binding proteins were found to be substrates for protein-O-carboxylmethyltransferase (PCM; E.C. 2.1.1.24). Calmodulin, an acidic calcium binding protein was originally thought to be a major substrate for PCM. Kinetic analysis revealed that only 5% of calmodulin could be carboxylmethylated, and that its Km for PCM was = 350 μM. Ca+2-dependent phosphodiesterase, calcineurin and Ca+2-calmodulin-dependent protein kinase were all carboxylmethylated with stoichiometric ratios of 0.2, 1.1, and 2.0 mol CH3/mol protein, respectively. Carboxylmethylation was found to reduce Ca+2-calmodulin dependent activities of each enzyme, with little effect on basal activities. Calcineurin, a Ca+2-calmodulin stimulated phosphatase, was the best substrate yet tested for PCM. These results suggest that PCM may regulate the calmodulin-stimulated activity of calcineurin, Ca+2-calmodulin protein kinase, and Ca+2-calmodulin-dependent phosphodiesterase.

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Objectives: Calmodulin-dependent binding proteins were observed to have a high capacity to accept methyl groups transferred by the enzyme protein-O-carboxylmethyltransferase (PCM). This study examined several homogeneous calmodulin binding proteins, namely, calcineurin, a calmodulin-dependent phosphatase, Ca^{+2}-calmodulin stimulated phosphodiesterase, and purified Ca^{+2}-calmodulin dependent protein kinase.

Methods: Methyl acceptor capacity and molar stoichiometry was estimated by protein, incubating the purified PCM, [^{3}H]methyl-S-adenosylmethionine, followed by precipitation and analysis of methyl group incorporation using 1) direct counting of the purified precipitate and 2) acidic SDS-gel electrophoresis. The calmodulin binding proteins phosphodiesterase and calcineurin were kindly provided by Dr. R. Kincaid, NHLBI.

In addition, substrate dependentcy analysis, time course, and methylester stability studies were conducted on the purified preparations, using direct analysis of methanol formed after acid precipitation followed by basic hydrolysis.

Enzyme activities for both phosphodiesterase (using cAMP as a substrate) and calcineurin (using para-nitrophenylphosphate as a substrate) were determined after carboxylmethylation.

Major Findings:

Phosphodiesterase: Ca^{+2}-calmodulin dependent phosphodiesterase was a substrate for PCM, incorporating up to 0.2 mol CH_3/mol enzyme. Phosphodiesterase activity was inhibited 30-50% by prior carboxylmethylation. This inhibition occurred after 1.0-2.0 min of carboxylmethylation. Basal activity was not significantly affected, suggesting that only calmodulin stimulated activity was inhibited. Acidic SDS-PAGE indicated that the M_r = 61,000 dalton protein corresponding to the subunit of homogeneous phosphodiesterase was carboxylmethylated. It was concluded that phosphodiesterase activity could be attenuated by carboxylmethylation with PCM.

Calcineurin: Calcineurin, a calmodulin-stimulated phosphatase, is the major calmodulin binding protein in brain. Calcineurin was carboxylmethylated in a time and S-adenosylmethionine-dependent manner. More importantly, calcineurin incorporated up to 2.0 mol CH_3/mol calcineurin, which is the highest stoichiometry yet seen for any substrate of PCM. Calcineurin carboxylmethylesters were quite labile in the native molecule, exhibiting a pH-dependent decrease in half life (pH 6.25 = 30 min; pH 7.0 ± 18 min; pH 7.5 = 3.0 min). In contrast, acid-precipitated carboxylmethylesters on calcineurin were more stabile, but still showed pH dependent decay (pH 6.25 = 42.0 min; pH 7.0 = 37.0 min; pH 7.5 = 26.0 min). Acidic SDS-PAGE indicated that virtually all of the methyl groups were incorporated on the A subunit (M_r = 61,000) of calcineurin. Enzyme activity studies indicated that when calcineurin was carboxylmethylated with at least 1.0 mol CH_3/mol protein, a significant inhibition of calmodulin-stimulated phosphatase activity was noted. Basal (Mn^{+2} supported) activity was not altered significantly. These results suggest that calcineurin can be directly carboxylmethylated by PCM, and when this occurs, calmodulin-stimulated phosphatase activity is inhibited.

Significance to Biomedical Research: The function of carboxylmethylation has remained enigmatic. The high stoichiometry of phosphodiesterase and calcineurin carboxylmethylation suggests that calmodulin binding proteins may be the prime substrates for PCM. In both cases, calmodulin-activated activity was inhibited, while basal activity was not affected. Thus, carboxylmethylation may modulate the activity of phosphodiesterase. More importantly, carboxylmethylation may also affect the tyrosine phosphatase activity of calcineurin.

Proposed Course of Research:

1. Physical biochemical studies will be undertaken to determine if carboxylmethylation alters the binding of calmodulin to carboxylmethylated calcineurin.

2. Calcineurin phosphatase activity will be determined after carboxylmethylation using a phosphotyrosyl protein substrate.

3. Attempts will be made to co-localize PCM and calcineurin in the CNS.

Publications

1. Billingsley, M., Kuhn, D., Velletri, P., Kincaid, R. and Lovenberg, W.: Carboxylmethylation of phosphodiesterase attenuates its activation by Ca^{+2}-calmodulin. J. Biol. Chem. 259: 6630-6635, 1984.

2. Billingsley, M.L., Kincaid, R.L. and Lovenberg, W.: Stoichiometric methylation of calcineurin by protein-O-carboxylmethyltransferase and its effects on calmodulin stimulated phosphatase activity. Submitted to J. Biol. Chem., 1984.

S-94

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03537-02 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Immunohistochemical Localization of Protein-O-Carboxylmethyltransferase in Brain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Melvin L. Billingsley	Staff Fellow	HE NHLBI
Other:	Donald Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Both biochemical and immunohistochemical techniques were used to demonstrate the presence of protein-O-carboxylmethyltransferase (PCM; E.C. 2.1.1.24) in the rodent CNS. The highest levels of immunoreactivity were detected in cortex, hippocampus, corpus striatum, thalamus and the amygdala. Other brain areas exhibited lower amounts of immunoreactive PCM. Most of the immunoreactive cells were neuronal with prominent labelling detected in both the cell body and the axonal region. Biochemical analysis of PCM activity correlated with the immunohistochemical localization. Methyl acceptor substrates for the enzyme were also high in regions rich in PCM. Western immunoblot analysis of bovine brain, rat brain and human erythrocyte forms of the enzyme showed the antisera generated against bovine brain PCM cross-reacted with human and rat forms of the enzyme, thus suggesting structural homology. These results suggest that PCM has an unique neuronal pattern of distribution, and that carboxylmethylation of proteins may be of functional significance in the nervous system.

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Objectives: PCM transfers methyl groups from S-adenosylmethionine (AdoMet) to free glutamic and/or aspartic acid residues on methyl acceptor protein (MAP) substrates. PCM activity is highest in brain, and has been postulated to participate in neuronal signal transduction. However, the distribution of PCM in brain has not been examined using histochemical methods. We have used an antisera generated against purified bovine brain PCM to show that PCM is localized in neurons throughout the brain, with highest levels found in the hippocampus, the striatum, the cortex and the thalamus. This work is a continuation of the project "Characterization of protein-O-carboxylmethyltransferase.

Methods: Antisera was generated in rabbits, using purified bovine brain PCM as an antigen. Antisera was collected, and an immunoglobulin fraction was partially purified by fractionation with 30% ammonium sulfate. Antisera in dilutions of 1:1000-1:5000 was incubated with 100 µM sections from formalin-fixed rat brain, using phosphate-buffered saline with 20% normal goat serum as a diluent. After incubation, sections were washed, incubated with a biotinylated goat-antirabbit IgG, and again washed with phosphate-buffered saline. An avidin-peroxidase complex was added, and the multiple antibody complex was visualized using diaminobenzidine as a substrate. In some experiments, PCM immunoreactivity was visualized using FITC-conjugated goat-antirabbit IgG, followed by viewing on a fluorescence microscope. Peroxidase-stained sections were mounted, cleared and dehydrated through a graded series of ethanol/xylene and viewed using bright-field illumination.

For western immunoblot analysis, PCM from bovine and rat brain and human erythrocyte was electrophoresed in the prescence of sodium dodecyl sulfate, and the proteins transferred to nitrocellulose paper. The excess protein binding sites were blocked using bovine serume albumin and hemoglobin, and antisera to PCM was incubated with the blot (1:5000). Immunoglobulin binding to PCM was detected using either the avidin/biotin/peroxidase system described earlier, or with goat-antirabbit-IgG coupled directly to horseradish peroxidase.

Biochemical analysis of PCM activity and MAP capacity was carried out using the alkali-induced release of ^{3}H-methanol from carboxylmethylesters as an index of PCM activity. When MAP capacity was examined, calcineurin, a calmodulin-dependent phosphatase, was used as a substrate.

Major Findings: PCM was localized in neurons throughout the rat brain. The hippocampus was prominently labelled, with pyramidal and granule cells in all regions showing immunoreactivity. The cortex also showed extensive immunoreactivity, and neurons in all layers (I-VI) appeared labelled. The striatum and thalamus also had substantial immunoreactive PCM present, while areas of the brainstem and cerebellum were only sparsely labelled.

Western immunoblot analysis indicated that antisera generated against bovine brain PCM could label both human erythrocyte and rat brain forms of the enzyme. All forms of the enzyme exhibit similar kinetic constants, amino acid compositions, and mobility on SDS-polyacrylamide gels. Thus, there is a strong likelihood that PCM is conserved between species.

Biochemical analysis of rat brain regions revealed that regions with high PCM immunoreactivity also had high activities of the enzyme. MAP capacity was

similarly distributed, again suggesting that the immunochemical localization corresponded with biochemical measures of protein carboxylmethylation.

Significance to Biomedical Research: This study marks the first time that PCM has been localized in regions of brain. The unique neuronal distribution correlated with biochemical activities, and suggests a functional role for PCM in neurons. The high degree of immune cross-reactivity, along with other biochemical similarities suggests that this enzyme is conserved among mammals. This study provides a strong basis for examination of the role of PCM in CNS activity.

Proposed Course of Project:

1. Neurochemical lesions of specific brain regions (6-hydroxydopamine; kainic acid) to produce specific anatomic changes will be performed and correlated with alterations in tissue PCM immunoreactivity.

2. Other antisera for neurotransmitters could be used to see if PCM is co-localized with any specific transmitter system.

3. The hippocampus will be used as a model brain region to study changes in PCM activity and content as a result of various pharmacologic manipulations.

Publications:

Billingsley, M.L., Kim, S., and Kuhn, D.M.: Immunohistochemical localization of protein-O-carboxylmethyltransferase in rat brain neurons. Submitted to Neuroscience.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03538-02 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Basic and Clinical Studies with Tetrahydrobiopterin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Walter Lovenberg	Laboratory Chief	HE NHLBI
Others:	Robert Levine	PRAT Fellow	IRP NIMH
	David Pickar	Psychiatrist	BP
	Peter LeWitt	Assistant Professor	Wayne State Univ.

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - X ☐ (a1) Minors
  - ☐ (a2) Interviews
- X ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Tetrahydrobiopterin (THB), the electron donor for aromatic amino acid hydroxylases plays a pivotal role in regulating the synthesis of biogenic amines. The current work addresses the question of regulation of THB in tissue, whether the level within a cell controls the rate of neurotransmitter synthesis and finally whether administration of this compound to subjects with cerain neurotransmitter deficits can affect neurological or behavioral activity. In cultured pineal gland, it was found that Methotrexate did not influence the THB synthesis. Together with other studies on intermediates in the BH4 biosynthetic pathway supportive evidence has been provided for the concept that the pterin ring structure is fully reduced through most of its biosynthetic steps. Other studies with PC-12 cells showed the THB levels were not coordinately regulated with tyrosine hydroxylase and that under some conditions tyrosine hydroxylase subunits could exceed the molecules of BH4.

Evaluation of therapeutic use of THB in Parkinson's disease, dystonia, and endogenous depression continued. Blinded evaluation of films of several patients with dystonia confirmed a major neurological improvement in individuals receiving up to 1 gm of THB. A second double blind study of 5 patients with endogenous depression failed to reveal significant clinical improvement. Although biochemical analysis showed that insufficient THB had penetrated the CNS.

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Objectives: The objective of our basic studies were as follows:

Studies were directed at further understanding the relationship between tyrosine hydroxyation and the availability of the hydroxylase cofactor. By examination of PC12 cells for tyrosine hydroxylase and cofactor under conditions which stimulate levels of tyrosine hydroxylase it should be possible to determine whether tyrosine hydroxylase and tetrahydrobiopterin are coordinately controlled.

The objective of another study was to further understand the biosynthetic mechanism by which GTP is converted tetrahydrobiopterin. The work was specifically devoted to the question of whether the final step in THB synthesis is catalyzed by the enzyme dihydrofolate reductase. Pineal glands were chosen for this study because they can be easily cultured and they contain a very high amount of BH_4. In this system we could evaluate the role of dihydrofolate reductase.

We also attempted to determine the ultimate clinical efficacy of tetrahydrobiopterin in various diseases where some positive responses have been noted, such as Parkinson's disease, dystonia, and endogenous depression. It is also important to understand why the clinical responsiveness is variable in the patient population.

Methods: PC-12 cells were kindly supplied by Dr. Gordon Guroff's laboratory and were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 5% horse serum in 10% CO_2 Dexamethasone was added to the medium in 0.01% ethanol (control medium lead similar addition of ethanol). Reduced and oxidized biopterin were analyzed by differential iodine oxidation and HPLC (Fukoshima and Nixon, Anal. Biochem. 102, 176, 1980). Tyrosine hydroxylase was monitored by a standard tritium release procedure.

Pineals from male Sprague-Dawley rats were cultured for up to 48 hours. Oxidized and reduced B were assayed by differential iodine oxidaton and high performance liquid chromatography (HPLC) (Fukushima and Nixon, Anal. Biochem. 102, 176, 1980). DHFR activity in pineal homogenates was assayed by measuring the formation of [^{3}H]-tetrahydrofolate from [^{3}H]-dihydrofolate (Hayman et al. Anal. Biochem. 87, 460, 1978).

In clinical studies, cerebrospinal fluid (CSF) was obtained by lumbar puncture before and after THB administration and analyzed for the content of biopterin (B) and the major metabolites of dopamine (DA) and serotonin (5-HT), homovanillic acid (HVA) and 5-Hydroxyindoleacetic acid (5-HIAA). B was assayed by high high-pressure liquid chromatography (HPLC) after oxidizing CSF with iodine under acid conditions. HVA and 5-HIAA were measured by electrochemical detection following HPLC. Clinical improvement in dystonia was monitored by standard techniques and video recordings were rated by independent neurologists in a blind fashion. The depressed patients were monitored for clinicl improvement using internationally accepted behavioral rating scales (AMP rating method global assessment scales).

Major Findings: Our major findings in the basic studies were given in last years annual report. To summarize briefly: Methotrexate did not inhibit the rate of THB synthesis in cultured pineal glands suggesting that dihydrobiopterin is not an intermediate in the THB biosynthetic pathway. This is consistent with other studies on the intermediates in THB synthesis in which it has been suggested that the first product after neopterin triphosphate is a diketopropyl-tetrahydropterin.

The work on PC-12 cells revealed that the levels of tyrosine hydroxylase subunits could be induced by dexamethasone treatment without concurrent increases in THB levels. In this study we observed that cells could contain more molecules of tyrosine hydroxylase than cofactor.

Finally, further evaluation of studies with the administration of THB to patients with familial dystonia reveal that this drug resulted in significant clinical improvement. Another clinical collaborative study is now nearing completion. Treatment of five patients with endogenous depression for two weeks with 1 to 2 g of THB (orally) resulted in minimal clinical improvement. Increments in catecholamine turnover could be demonstrated in the periphery. However, analysis of cerebrospinal fluid indicated that with this route of administration insufficient material penetrated the blood brain barrier to cause an enhancement of biogenic amine synthesis. Further studies are planned.

Significance to Biomedical Research: A number of human diseases may be related to an over or under activity specific types of neurons. An understanding of the fundamental regulatory properties and ways to manipulate the activity of certain specific types of neuron will lead both to better understanding of the system and potentially new therapeutic approaches. In the current study we have pursued an earlier observation suggesting that tetrahydrobiopterin levels are low in some patients with familiar dystonia. Several patients have shown significant neurological improvements with this treatment.

Proposed Course of Project: A large clinical trial with BH_4 is planned in collaboration with NIMH to determine the merits of long-term treatment of depressed patients. New patients with dystonia will also continue to be treated with BH_4 in collaboration with Peter LeWitt at Wayne State University and neurologist in NINCDS. The collaboration with Curtius and Niederwieser in Zurich will focus on ways of enhancing BH_4 entry into brain from the periphery by coupling BH_4 to molecules which are actively transported into brain.

Publications:

Levine, R.A., Lovenberg, W., Curtius, H.-Ch., and Niederwieser, A.: Penetration of reduced pterins into rat brain: Effect on biogenic amine synthesis. In: Pteridines and Folic Acid Derivatives, Walter de Gruyter, Berlin, New York, 1982, in press.

LeWitt, P., Miller, L., Insel, T., Calne, D., Lovenberg, W., Levine, R. and Chase, T.: Tyrosine hydroxylase cofactor (tetrahydrobiopterin) in parkinsonism. Advances in Neurology 33, in press.

Levine, R.A. and Lovenberg, W.: Speculation on the mechansim of therapeutic action of tetrahydrobioptern in human disease. In: Pteridines and Folic Acid Derivatives, Walter de Gruyter, Berlin, New York, 1983, p. 833.

Curtius, H.-Ch., Niederwieser, A., Levine, R. and Muldner, H.: Effectiveness of tetrahydrobiopterin in the treatment of Parkinson's disease. Advances in Neurology 33, in press.

Curtius, H.-Ch., Hausermann, M., Heintel, D., Niederwieser, A., and Levine, R.A.: Perspectives on tetrahydrobiopterin biosynthesis in mammals. In: Pteridines and Folic Acid Derivatives, Walter de Gruyter, Berlin, New York, 1983, in press.

Lovenberg, W., Levine, R.A., Aiken, M.A., Miler, L.P. and LeWitt, P.: Regulation of Biogenic Amine Synthesis by the Hydroxylase Cofactor and its Relation to Aging and Parkinsonism. In: Intervention in Aging Publication Based on FIBER's Conference on Aging, Boston, MA, October 1982, W. Regelson and M. Sinex, Eds., A. Liss, New York, pp. 215-224.

Culvenor, A., Miller, L., Levine, R., and Lovenberg, W.: Effects of methotrexate on biopterin levels and synthesis, in rat cultured pineal glands. J. Neurochem., in press.

Culvenor, A., Zabrenetsky, V. and Lovenberg, W.: Effects of dexamethasone on biopterin levels and the tyrosine hydroxation system in PC-12 cells. Biochem. Pharmcol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03539-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Serotonergic Innervation of the Nucleus Tractus Solitarius.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donald M. Kuhn	Pharmacologist	NHLBI HE
Others:	William A. Wolf	Guest Worker	NHLBI HE
	Ulrike Berresheim	Visiting Scientist	NHLBI HE

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The fluorescent dyes Propidium Iodide (PI) and True Blue (TB) are transported along axons in a retrograde direction after microinjection of these substances in brain. Injection of PI or TB in the nucleus tractus solitarius (NTS) followed by fluorescence histochemical processing 3-7 days later revealed that the dyes are transported to the dorsal raphe nucleus. Concurrent immunohistochemical analysis indicated that the dye was present in cells of the dorsal raphe which also contain serotonin. Few other areas in the dorsal mesencephalon were labeled with dye whereas the pons did show some dye. These results suggest that the origin of the serotonergic innervation of the NTS is the dorsal raphe nucleus.

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Objectives: The nucleus tractus solitarius (NTS) is a dorso-medial medullary nucleus and is the site where primary afferents of the baroreceptor pathway first synapse. The chemical transmitter which modulates the baroreceptor reflex at the level of the NTS is not known but the NTS contains very high levels of serotonin (5-HT). Furthermore, microinjections of 5-HT into the NTS produce transient pressor effects in rats (Wolf, Kuhn, and Lovenberg, Eur. J. Pharm. 69: 291-299, 1981). The first step in learning how a brain structure might function involves the delineation of its chemical anatomy. Therefore, the objective of these studies was to determine the site of origin of the nerve endings in the NTS which contain 5-HT.

Methods: Male Sprague-Dawley rats were anesthetized and placed in a stereotoxic apparatus. The dorsal surface of the medulla was exposed by a partial laminectomy and the atlanto-occipital membrane was carefully retracted. The dyes TB or PI were injected into the NTS by visually placing a single-barrel micropipette containing either dye 1.0 mm lateral to the obex and 1.0 mm ventral to the surface of the medulla. Injections of 1.0 µl were made over a 15 min time period.

After a survival time of 3-7 days, rats were perfused transcardially with 4% formaldehyde and serial 50 µ sections were subsequently cut through the mesencephalon. Immunohistochemical processing was carried out by incubating free floating sections with primary antiserum (anti-serotonin) diluted 1:500. After washing, a secondary antibody crosslinked with FITC was added to the sections. Finally, sections were mounted on slides, coverslipped, and viewed on a Leitz Dialux EB20 fluorescence microscope equipped with epi-illumination.

Major Findings: Within 3 days of injection of either TB or PI into the NTS, the dyes could be observed in the area of the dorsal raphe nucleus. The median raphe was not labeled to any significant extent. If injections of dye were superficial in the medulla, the pons was subsequently labeled very densely. Concurrent immunohistochemical analysis indicated that many of the cells in the dorsal raphe also contained serotonin.

Significance to Biomedical Research and Institute Programs: These results establish that at least part of the serotonergic innervation of the NTS arises in the dorsal raphe nucleus. This result is somewhat surprising since it is generally thought that the dorsal raphe projects in an ascending direction. However, since the NTS contains nearly the highest levels of 5-HT in the brain, one might expect that the 5-HT innervation of the NTS would arise from a large 5-HT containing cell body area. Furthermore, since injections of 5-HT into the NTS produce a pressor effect in rats and since electrical stimulation of the dorsal raphe also leads to pressor effects, the anatomic connection from the raphe nucleus to the NTS probably has functional significance in the reflex control of the cardiovascular system.

Proposed Course of Project: In order to more clearly establish that the raphe sends 5-HT projections to the NTS, the raphe nuclei will be destroyed by electrocoagulation. After 2-4 weeks, the levels of 5-HT in the NTS and other medullary sites will be determined as previously described (Wolf and Kuhn, J. Chromatog. 275: 1-9, 1983). If the raphe is the source of NTS 5-HT, destruction of the raphe with consequent anterograde degeneration should reduce NTS 5-HT levels.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03540-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Release of Serotonin: Evidence in Support of Cytoplasmic Release.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William A. Wolf	Guest Worker	NHLBI HE
Other:	Donald M. Kuhn	Pharmacologist	NHLBI HE
	Moussa B.H. Youdim	Visiting Scientist	NHLBI HE

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The in vivo release of serotonin (5-HT) was investigated by studying a behavioral/neurological syndrome in rats. The syndrome is referred to as the 5-HT syndrome and is comprised of a variety of symptoms including head weaving, forepaw treading, hindlimb abduction, and tail-lashing. The syndrome was presently studied in rats which had been treated with reserpine to abolish the vesicular pool of 5-HT. Injections of the 5-HT releasing drug parachloroamphetamine (PCA) produces the entire syndrome in reserpinized rats just as it does in normal rats. Parachlorophenylalanine, which inhibits the synthesis of 5-HT, or metergoline, the 5-HT receptor antagonist, each prevent the PCA effect in reserpinized rats indicating that the 5-HT remaining after reserpinization represents a newly synthesized cytoplasmic pool of transmitter. Increases in this cytoplasmic pool with injections of a non-selective monoamine oxidase inhibitor or with a selective inhibitor of MAO-A, but not with a selective inhibitor of MAO-B, markedly reduces the dose of PCA necessary to produce the 5-HT syndrome. Thus, increases or decreases in the cytoplasmic pool of 5-HT can increase or decrease in vivo release, respectively, induced by PCA.

Objectives: The neuronal mechanisms controlling the release of the neurotransmitter serotonin are poorly understood. Serotonin is stored in two separate pools in brain and it has been assumed that the transmitter is released by exocytosis. However, evidence in support of the exocytotic release of 5-HT is sparce. In an effort to learn more about 5-HT release in vivo, we have studied a behavioral syndrome which has been shown to be a specific and reliable assay for 5-HT release in vivo. Furthermore, these experiments were carried out in reserpinized rats. We have assumed that release of 5-HT in a reserpinized rat would occur from the cytoplasm since reserpine abolishes the vesicular pool of 5-HT.

Methods: Male Sprague-Dawley rats were injected with 5.0 mg/kg reserpine 24 hr prior to testing. After test injections, rats were observed and scored for expression of the characteristic symptoms of the 5-HT syndrome (i.e., head weaving, forepaw treading, hindlimb abduction, and tail lashing). All animals were sacrificed at the appropriate times and brain areas were assayed for 5-HT as described by Wolf and Kuhn (J. Chromatog. 275: 1-9, 1983).

Major Findings: Reserpine causes a large reduction in 5-HT levels. Within 24 hr of reserpine, 5-HT is reduced throughout the brain and spinal cord by 90-95%. Despite this reduction in 5-HT, the 5-HT releasing drug parachloroamphetamine (PCA) could still elicit the entire 5-HT behavioral syndrome. PCPA, which inhibits 5-HT synthesis, and metergoline, a 5-HT receptor blocker, each prevented the PCA-induced syndrome. Treatment of reserpinized rats with a nonselective inhibitor of MAO increases 5-HT levels almost 10 fold. This treatment drastically reduces the dose of PCA necessary to produce the 5-HT syndrome. The selective MAO-A inhibitor clorgyline also shifts the PCA dose-response curve to the left while deprenyl, a selective MAO-B inhibitor, does not.

Significance to Biomedical Research and Institute Programs: These results establish that the release of 5-HT can occur even when the vesicular stores of this transmitter have been abolished. Increases or decreases in the remaining cytoplasmic pool of 5-HT produces a corresponding increase or decrease in release. These data minimize a role for exocytosis in mediating 5-HT release and suggest that the releasable or functional pool of 5-HT is cytoplasmic.

Proposed Course of Project: The cytoplasmic levels of 5-HT will be manipulated by various pharmacological means in an attempt to establish a closer correlation between the cytoplasmic pool and release. Furthermore, correlative experiments on 5-HT release will be carried out in isolated synaptosomes prepared from normal or reserpinized animals.

Publications:

Kuhn, D.M., Murphy, D.L., and Youdim, M.B.H.: Physiological and clinical aspects of monoamine oxidase. In Mondovi, B. (Ed.), Structure and Function of Amine Oxidases. New York, CRC Press, 1984, in press.

Kuhn, D.M. and Youdim, M.B.H.: The neuropharmacology of serotonin: Functional pools of transmitter and their regulation by monoamine oxidase. In Bunney, B. and Barchas, J. (Eds.) Frontiers in Neuropsychopharmacology. New York, Alan Liss, 1984, in press.

Youdim, M.B.H., Finberg, J.P.M., Kuhn, D.M., and Wolf, W.A.: The role of monoamine oxidase A in the metabolism and function of noradrenaline and serotonin. Brit. J. Pharmacology, 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03541-01 HE

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Vascular Smooth Muscle Cells in Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toru Nabika	Visiting Fellow	HE NHLBI
Other:	Paul Velletri	Staff Fellow	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation of cAMP and Ca++ accumulation by vascular smooth muscle cells (SMC) grown in culture from aortas of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) was explored. No differences in the rate or extent of cAMP or Ca++ accumulation were observed when SMC from WKY, SHR or stroke-prone SHR were compared. Beta-agonists, and to a lesser extent vasoactive intestinal polypeptide, forskolin, PGE1 and PGE2, increased cAMP levels. Of all treatments studied, only angiotensin II and high extracellular K+ were capable of enhancing Ca++ uptake. Exposure of SMC and vascular fibroblasts (used as control vascular cells) to dibutyryl cAMP led to morphological changes in both cell types characterized by cytoplasmic shrinking and cellular elongation. However, only SMC exhibited morphological changes following exposure to β-agonists, which were potent stimulators of cAMP production in SMC, but not in fibroblasts. Colchicine reversed the morphological changes associated with cAMP accumulation, suggesting the participation of cellular microtubules in cytoplasmic shrinking. These studies have allowed us to develop a useful model system to study the cellular biology of SMC and the biochemical differences between SMC derived from WKY and SHR in a milieu isolated from the complexities of the intact organism.

PHS 6040 (Rev 1/84) GPO 904-917

Objectives: Our research has attempted to clarify mechanisms by which vascular smooth muscle cells (SMC) regulate the levels of intracellular second messengers, and to understand the relationship between these messengers and the physiological functions of the cells. We have attempted to delineate how SMC control the turnover of cyclic nucleotides (in particular cyclic AMP) and intracellular free calcium (Ca^{++}) as a response to a number of well-characterized vasoactive substances. Furthermore, we have studied whether there exist differences in the regulation of cAMP or Ca^{++} levels when values in SMC derived from normotensive Wistar-Kyoto (WKY) rats and their spontaneously hypertensive (SHR) counterparts were compared.

These studies have been conducted in cell culture from cells explanted from age-matched WKY and SHR. The use of cultured vascular SMC allows us to observe biochemical and pharmacological events in cells that have been isolated from the potential factors to which SMC might be exposed in vivo, such as blood pressure and neuronal or humoral influences. Hence, phenomena that are observed in culture may be thought to be the result of intrinsic cellular factors and not the result of physiological events unrelated to the biology of SMC.

Methods:

(i) SMC Culture: SMC were derived from thoracic aortas of WKY, SHR and stroke-prone SHR (SHRSP) by the explantation method described by Ross (JCB 50: 172, 1971). Cultured SMC were grown in culture flasks and the medium changed every three days. Cells were transferred to new flasks at confluence (every 7-10 days). In cell experiments, cells of 3-12 passages were used.

(ii) cAMP Determinations: Confluent cell cultures (approximately 1×10^6 cells/dish) were incubated at 37°C in the presence of a variety of vasoactive substances for different periods of time (see results). At the end of the incubation periods, intracellular cAMP was extracted into perchloric acid (PCA) at 4°C. Cyclic AMP in the PCA extracts (supernatant fraction) was quantified with the use of a radioimmuoassay kit obtained from New England Nuclear. The residual pellet was used as a source of protein, which was determined by the method of Bradford (Anal. Biochem. 72: 248, 1976). The pellet was solubilized in 1N NaOH prior to assay.

(iii) $^{45}Ca^{++}$ Accumulation: Confluent cell cultures were preincubated at 37°C in the presence of $^{45}CaCl_2$ (1-5 μM) simultaneously or prior to determining the effects of vasoactive substances on $^{45}Ca^{++}$ influx and efflux. Incubations were terminated by the removal of the medium containing $^{45}Ca^{++}$. The cells were then washed with a Ca^{++}-free buffer containing La^{3+}, which has been shown to block extracellular Ca^{++}-binding sites. Cells were digested with 1N NaOH and aliquots used to count radioactivity. Ca^{++} uptake was expressed either on a mg protein or cell number basis.

Major Findings:

(i) Regulation of cAMP Levels: SMC from WKY, SHR and SHRSP were capable of accumulating cAMP in the presence or absence of the phosphodiesterase inhibitor, theophylline, following exposure to a variety of biologically active agents. Accumulation was higher, more rapid and more prolonged in the presence of

6 CP

theophylline. Cyclic AMP accumulation following exposure to β-agonists such as isoproterenol could be enhanced almost 300-fold. The stimulation of cAMP accumulation by isoproterenol was completely blocked by β-antagonists such as propranolol. Alpha-agonists such as phenylephrine had no effect on cAMP levels. Vasoactive intestinal polypeptide (VIP), forskolin, PGE_1 and PGE_2 also were able to increase cAMP levels in SMC, but were not as potent as isoproterenol. Interestingly, there were no apparent differences in the response of SMC derived from WKY, SHR and SHRSP to β-receptor stimulation. Furthermore, basal cAMP levels of each line of SMC were similar.

Fibroblasts isolated from rat aorta also responded to β-receptor stimulation, but the extent of cAMP accumulation was considerably lower than that of SMC. Exposure of SMC and fibroblasts to dibutyryl cAMP stimulated morphological changes in both cell types. These morphological changes were characterized by cellular elongation and cytoplasmic shrinking, events that led to the appearance of "cellular processes." However, only SMC responded to isoproterenol, suggesting that poor coupling of cAMP production to β-adrenergic stimulation in fibroblasts prevented the morphological alterations observed following exposure to dibutyryl cAMP. Colchicine prevented cAMP-induced morphological changes in SMC and fibroblasts, indicating a role for microtubules in the morphological events.

(ii) $^{45}Ca^{++}$ Accumulation: Of all vasoactive agents employed, only angiotensin II and elevated concentrations of extracellular K^+ were capable of increasing the extent of $^{45}Ca^{++}$ accumulation in all lines of SMC. Surprisingly, not all vasopressors (phenylephrine, prostaglandins) tried were capable of altering $^{45}Ca^{++}$ accumulation. No differences among SMC from WKY, SHR and SHRSP were noted with regard to basal or stimulated $^{45}Ca^{++}$ accumulation.

Significance to Biomedical Research and Institute Programs: The studies described above have allowed us to establish a useful model system for the study of the pathogenesis of hypertension. The culturing of vascular SMC allows us to study indirectly the abnormalities in contractility that are the final common denominators of high blood pressure. Our ability to culture SMC and to study the regulation of second messengers involved in contractility will allow us to study the cell type (i.e., SMC) most responsible for the elevated blood pressure in hypertension. SMC can now be studied when isolated from whole animal factors such as blood pressure and neuronal or humoral influences. The goals of our research are in keeping with the general objectives of the Hypertension-Endocrine Branch and will allow us eventually to study numerous aspects of the cell biology of normo- and hypertensive SMC.

Our pilot studies have demonstrated the cAMP can accumulate following β-adrenergic stimulation and that this response is primarily associated with one vascular cell type (SMC), and not the pervasive fibroblasts. Beta-agonists are well known to cause vascular relaxation of SMC. Our pilot studies have also demonstrated that K^+ and angiotensin II cause an enhanced accumulation of Ca^{++} in SMC, a phenomenon associated with vasoconstriction. To date, no differences in the responsiveness of SMC derived from WKY, SHR or SHRSP have been observed with any parameter assayed.

Little is understood about the regulation of vascular contractility in either normal homeostatic or pathological conditions. Related to mechanisms of contractility are: (1) receptor-binding of vasoactive substances to the plasmalemma of SMC; (2) membrane transduction mechanisms; (3) the generation of intracellular second messengers, such as cAMP; (4) increases in the membrane's permeability to ions, such as Ca^{++}; and (5) a combination of physical and biochemical events resulting in muscular contraction. We have chosen to commence our studies on the cell biology of SMC derived from normal and hypertensive animals by delineating control mechanisms for the regulation of two second messengers -- cAMP and Ca^{++}. Both have been implicated in playing a significant role in the pathogenesis of abnormalities of contractility in SMC of hypertensive individuals.

The heterogeneity of vascular tissue (i.e., endothelium, SMC, fibroblasts, etc.) has made certain conclusions with regard to SMC in intact tissue difficult to interpret in the past. Our observations that SMC appear to accumulate cAMP more extensively than the pervasive fibroblasts suggest that studying an individual cell type may lead to novel insights on the regulation of second messengers in SMC.

Proposed Course of Project: Within the next year, we anticipate studying in more detail the role of cAMP and Ca^{++} in vascular smooth muscle physiology and pathology. We will attempt to relate the cellular regulation of free cAMP and Ca^{++} levels to the pathogenesis of hypertension by comparing the cAMP and Ca^{++} regulatory systems of SMC from WKY and SHR. In particular, we anticipate studying how vasoactive substances modulate the production of cAMP that results from β-stimulation and exploring mechanisms by which free cytosolic Ca^{++} levels are controlled by vasopressor agents such as angiotensin II. These latter studies will employ novel and sensitive techniques for the measurement of free Ca^{++} by using the fluorescent Ca^{++} indicator, quin 2. These studies are being undertaken with the collaboration of Dr. Michael Beavan of the Laboratory of Chemical Pharmacology.

Publications:

Nabika, T., Velletri, P.A., Igawa, T., Yamori, Y. and Lovenberg, W.: Comparison of cyclic AMP accumulation and morphological changes induced by -adrenergic stimulation of cultured vascular smooth muscle cells and fibroblasts. Blood Vessels, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03542-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Phosphatidylinositol Pathway in Cultured Aortic Smooth Muscle Cells.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen Buck	PRAT Fellow	HE NHLBI
Other:	Toru Nabika	Visiting Fellow	HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mn++ stimulates 3H-inositol incorporation into membrane phosphatidylinositol in cultured aortic smooth muscle cells from WKY and SHR rats. The stimulation does not occur with other cations and appears to result from activation of enzymatic exchange of free inositol with the headgroup inositol of membrane PI. The Mn++-stimulated 3H-inositol incorporation is lower in SHR cells than in WKY cells suggesting a biochemical defect in SHR aortic smooth muscle cells. This defect may reside in the endoplasmic reticulum of the smooth muscle cells.

611

GPO 904-917

Objectives: Stimulation of the turnover of the phospholipid, phosphatidylinositol (PI), is believed to be linked to neurotransmitter and hormonal activation of Ca^{++} influx in membranes. PI and/or its phosphorylated metabolites (i.e., PI-4-PO_4; PI-4,5-bis PO_4; inositol 1-PO_4; inositol 1,4-bis PO_4; inositol 1,4,5-triPO_4) are thought to be directly linked to this Ca^{++} gating mechanism. Activation of Ca^{++} influx has been linked to synaptic function in the CNS and to secretion and smooth muscle contraction in the periphery. We have undertaken an investigation of the PI pathway in cultured, dissociated smooth muscle cells from aortae of WKY and SHR rats.

Methods: Harvested cells are incubated in Kreb-HEPES buffer at 37°C for 45 min to 2 hr in the presence of ^{3}H-inositol and test stimulatory agents. The ^{3}H-inositol incorporated into lipid is extracted into chloroform in the presence of methanol and water, the chloroform layer is withdrawn and dryed, and the residual radioactivity is then determined.

Major Findings: In the absence of Mn^{++}, the basal incorporation of ^{3}H-inositol into PI is very slow even up to 2 hr of incubation time. Inclusion of Mn^{++} in the assay results in a 10- to 20-fold increase in ^{3}H-inositol incorporation. The incorporation is linear with increasing number of cells and with increasing amounts of ^{3}H-inositol. Under standard assay conditions (0.25×10^6 cells, 0.25×10^6 CPM, 45 min), the EC_{50} for Mn^{++} is 1.0 mM. The incorporation is temperature-dependent, inhibited by dinitrophenol (IC_{50} = 0.1 mM), and Mn^{++} is not mimicked by Ba^{++}, Zn^{++}, Sr^{++}, La^{+++}, Ni^{+}, Ca^{++}, Mg^{++}, Co^{++}, or Li^{+}. We have found that cells from the aortae of SHR rats have a decreased responsiveness to Mn^{++} compared to cells cultured from aortae of WKY animals. Approximately 75% of the SHR cell lines exhibit a maximum Mn^{++}-stimulated ^{3}H-inositol incorporation that is 50% of that of WKY lines. Serotonin (5-HT) and angiotensin II, but not carbachol, also stimulate ^{3}H-inositol incorporation into PI in the cultured smooth muscle cells.

Significance to Biomedical Research and Institute Programs: This is the first investigation of the PI pathway in cultured smooth muscle cells. We have determined that there is a possible biochemical defect in SHR cultured cells in their responsiveness to Mn^{++}-stimulation of ^{3}H-inositol incorporation into PI. The potential defect may provide clues to the source of abnormal Ca^{++} dynamics in SHR cells and to any genetic mechanism underlying the defect.

Proposed Course of Project: Two aspects of the above observations will be pursued at this time:

1) A similar Mn^{++}-activated inositol incorporation has been observed in endoplasmic reticulum from liver of normal rats. The possibility that the apparent Mn^{++}-stimulated enzyme activity we have observed in cultured smooth muscle cells is associated with endoplasmic reticulum will be considered, as will the possibility that there is some defect in this organelle in SHR cells.

2) The effects of neurochemicals on ^{3}H-inositol incorporation and on PI turnover in plasma membrane will be compared in WKY and SHR cell lines to determine if the defect in Mn^{++}-stimulation in the latter is accompanied by abnormalities in PI metabolism in the membrane.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03543-01 HE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Multiple Types of Tachykinin Receptors.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen Buck	PRAT Fellow	HE NHLBI
Others:	Elizabeth Burcher	Visiting Scientist	ET NINCDS
	Clifford W. Shults	Medical Staff	ET NINCDS
	Thomas L. O'Donohue	Unit Head	ET NINCDS
	Mei Lie Swenberg	Research Chemist	HE NHLBI

COOPERATING UNITS *(if any)*

Neuroendocrinology Unit, Experimental Therapeutics Branch, NINCDS

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

3H-Substance P (3H-SP), 3H-eledoisin (3H-ELE), and 125I-substance K (125I-SK) have been utilized to examine the characteristics of tachykinin binding sites in mammalian tissues. 3H-SP, 3H-ELE, and 125I-SK binding in rat salivary gland membranes is inhibited by tachykinins in the rank order of SP≥physalaemin>SK>ELE>kassinin which is consistent with the existence of only a SP-P type receptor in this tissue. In smooth muscle membranes from guinea-pig small intestine and rat duodenum, 125I-SK binding is inhibited in the rank order of SK>ELE>kassinin>SP≥physalaemin which is distinct from SP-P type binding and from the SP-E type receptor suggested by in vitro bioassays. The gut appears to contain a unique SK binding site which may be the receptor for SK released from neurons in which the peptide coexists with SP. There is an asymmetric distribution 125I-SK binding sites throughout the rat gastrointestinal tract and the rat has substantially more of these sites in the pylorus, fundus, duodenum, and colon than does the guinea pig, 125I-SK also binds to sites in the rat brain. 3H-SP binds presumably to the SP-P receptor in rat brain and the levels of this binding are transiently elevated during the last prenatal and first postnatal week. 3H-SP also binds to high affinity sites in bovine adrenal cortex and medulla. We have been unable to demonstrate binding of 3H-SP, 3H-ELE, or 125I-SK to intact cultured cells from WKY and SHR rat aortae.

Objectives: The putative peptide neurotransmitter, substance P (SP), is a member of a family of peptides known as tachykinins. Members of this family contain the C-terminal sequence Phe-X-Gly-Leu-Met-NH_2 and include SP and the non-mammalian peptides physalaemin (PHYS), eledosin (ELE), and kassinin (KAS). Until recently, it was thought that SP was the only mammalian tachykinin. SP is suspected of being a neurotransmitter or neuromodulator in basal ganglia and other brain regions; in sensory neurons of spinal ganglia that mediate pain, temperature sensation, certain cardiovascular reflexes, and probably other visceral sensory functions; and in the gastrointestinal tract. SP is a potent hypotensive agent, salivary secretagogue, and contractor of visceral smooth muscle. Physalaemin-like immunoreactivity has now been found in low levels in various mammalian tissues and in relatively high levels in a small-cell carcinoma of human lung. In addition, the interesting discovery was recently made that SP and a kassinin-like peptide that has been named substance K (SK) coexist in a precursor molecule in bovine corpus striatum. Based on bioassay data, it has been proposed that there are two distinct types of SP receptors in mammalian tissues: a SP-P receptor which has a higher affinity for SP and PHYS than for ELE or KAS, and a SP-E receptor which has a higher affinity for ELE and KAS than for SP or PHYS. With the use of novel radiolabeled ligands, we have begun to characterize the binding characteristics of tachykinins in tissue membrane fragments. Part of this work is being carried out in collaboration with investigators in the NINCDS who are studying the autoradiographic distribution of tachykinin receptors.

Methods: Tissues are homogenized in 50 mM TRIS buffer and centrifuged at 48,000 x g. The pellets are then resuspended for 1 hr in TRIS containing 10 mM EDTA to inactivate metallopeptidases. After several washings, the crude membranes are resuspended in an assay buffer consisting of TRIS containing BSA, chymostatin, leupeptin, bacitracin, and Mn^{++}. An optimal amount of tritiated or iodinated tachykinin ligand is included to give total binding and the difference between this and the binding in the presence of an excess of unlabeled peptide (non-specific binding; usually 1 μM unlabeled compound) is defined as specific binding. Binding characteristics such as optimal tissue concentration, time of equilibrium, reversibility, and rank order potency of other competitors are then determined for the ligand. Distribution of binding and comparisons between species and different aged animals can then be carried out.

Major Findings: ^{3}H-Eledoisin was synthesized for use in characterizing SP-E receptor binding. The specific activity of the compound was 30 Ci/mmole. A substantial amount of ^{3}H-ELE binding was observed in rat salivary gland and gut smooth muscle with trivial amounts observed in heart, lung, brain, and urinary bladder. The binding in the salivary gland exhibited characteristics of an SP-P type site indicating that ^{3}H-ELE at the nanomolar concentrations required for sufficient counts bound will label SP-P receptors. This may indicate that ^{3}H-eledoisin will be useful only in tissues with a preponderance of SP-E receptors. In order to characterize the nature of SK binding, Bolton-Hunter-^{125}I-SK was prepared with a specific activity of 1800 Ci/mmole. A high amount of ^{125}I-SK binding was observed in salivary gland, gut, and brain. Using a low concentration of ^{125}I-SK (i.e., 100 pM), the binding of this ligand in salivary gland was exclusively to a SP-P type site. This observation and the results with ^{3}H-eledoisin in the gland apparently indicate that rat salivary gland contains only a SP-P receptor. In smooth muscle of the guinea pig small intestine and of the

rat duodenum, the rank order of competition for binding of 100 pM ^{125}I-SK was SK>ELE>KAS>SP>PHYS. This pattern is not that of a SP-P type site nor does it correspond to that of a SP-E type of binding seen in bioassays where SK, ELE, and KAS are all essentially equipotent. This pattern of SK binding in the gut appears to be unique and may represent a third type of tachykinin receptor, a K receptor. The amount of ^{125}I-SK binding is identical throughout most of the small intestine of the rat and the guinea pig whereas the rat pylorus, fundus, duodenum, and colon contain 10- to 100-fold higher levels of binding of the ligand. Characterization of ^{125}I-SK binding in rat brain is underway. We have also determined that ^{3}H-SP can be used to label SP-P sites in the brain of prenatal, neonatal, and adult rats. Preliminary results indicate that there are more of these sites during the last week of gestation and during the first postnatal week than in adult animals. SP may have an important function in the late development of the brain. ^{3}H-SP also binds to bovine adrenal cortical and medullary membrane fragments. The pharmacological characterization of this binding has not been completed, but the SP receptors in the adrenal gland may be linked to the little known sensory afferent innervation of the gland and may mediate the reported inhibition by SP of cholinergic-stimulated adrenal catecholamine release. Radiolabeled SP, SK, and ELE do not bind to cultured, dissociated smooth muscle cells from rat aorta.

Significance to Biomedical Research and Institute Programs: The existence of a distinct receptor for SK would be an important finding as it would mean that there are separate receptors for each of two coexisting tachykinin neuropeptides. This situation has been described before only for the opioid peptides Met- and Leu-enkephalin. Since SP is thought to act on SP-P type receptors to activate phosphatidylinositol turnover and Ca^{++} influx, the existence of a separate receptor for SK could indicate a different second messenger system for the latter peptide. From time to time, investigators have postulated that SP-like neuropeptides have an important role as neurotrophic agents instead of or in addition to the neurotransmitter role. Elucidation of SK receptors may clarify whether neurotransmission or neurotrophism is the more important function of the various mammalian tachykinins. Comparison of the relative levels and distribution of SP and SK binding sites may provide information about the importance of each of these agents in the different functions that may be served by SP-containing neurons in the brain, gastrointestinal tract, and cardiovascular systems.

Proposed Course of Project: Several avenues of investigation will be pursued in this project:

1) The binding of ^{125}I-SK in gastrointestinal smooth muscle will be compared to that of ^{125}I-ELE in the same tissue to ascertain if SK is binding to a site other than the SP-E type.

2) The characterization of the binding of ^{125}I-SK in brain will be completed.

3) ^{3}H-SP binding in bovine adrenal gland will be investigated. In addition, if PC_{12} cells in culture become available in the Section, the binding of ^{3}H-SP in these cells will be investigated. SP has been reported to inhibit cholinergic-stimulated dopamine release in PC_{12} cells.

4) The time course of the onset of adult-level ^{3}H-SP binding in the developing rat brain will be completed. This investigation will focus on several prenatal, neonatal, and adult ages of animals and the development of ligand binding will be compared to age-related changes in levels of brain SP immunoreactivity. Tissue preparations from whole brain, as well as from several brain regions, will be considered.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 to September 30, 1984

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that control transport.

Isolated segments of renal tubules.

In order to understand kidneys at a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings, during the past year, using this method are as follows:

Knepper, Tomita, and Pisano investigated the effects of mineralocorticoids, arginine vasopressin, and bradykinin on electrolyte transport by isolated perfused cortical collecting ducts from rats. There was no detectable sodium or potassium transport across collecting ducts from untreated animals. Chronic administration of deoxycorticosterone to the rats increased sodium absorption and potassium secretion by their collecting ducts perfused in vitro. Addition of arginine vasopressin to the bath in vitro increased both sodium and potassium transport above the deoxycorticosterone-stimulated levels. In contrast, addition of bradykinin to the bath in vitro inhibited sodium absorption, but did not significantly affect potassium transport. The collecting ducts are known to be important for controlling sodium and potassium excretion, and the kallikrein-kinin system was believed to be involved. These studies are the first to demonstrate the exact renal effect of kinin and they are elucidating the complex interrelationship between the many hormones that affect the collecting ducts.

Knepper, Burg, Good, and Garcia-Austt studied ammonia and bicarbonate transport by isolated, perfused thick ascending limbs and cortical collecting ducts from rats and rabbits. Thick ascending limbs from rats absorbed ammonia. The mechanism is passive NH_4^+ transport driven by the lumen positive transepithelial electrical potential difference. The rat thick ascending limbs also absorbed bicarbonate. The mechanism is secondary active secretion of protons, mediated by sodium-proton exchange across the apical membrane of the epithelial cells. Neither transport process had been known before these studies. The ammonia transport is important because it results by countercurrent multiplication in the high concentration of ammonia in the medulla which is necessary for efficient excretion of ammonia during antidiuresis. The bicarbonate transport is important because it is large in extent and represents a major sodium-dependent mode of acidification a distal tubule segment.

Cortical collecting ducts, on the other hand, secreted large amounts of bicarbonate actively when the rats or rabbits from which they were taken had been chronically treated with deoxycorticosterone. This stimulation of bicarbonate secretion was prevented by giving the animals a sufficient intake of acid to prevent the metabolic alkalosis ordinarily caused by deoxycorticosterone. Thus, the bicarbonate secretion apparently is a response to the alkalosis, rather than to the deoxycorticosterone per se. Based on this and previous evidence, the cortical collecting duct is an important site of alkalinization of the urine. The bicarbonate secretion required chloride in the perfusate. The rate increased with a lumen-to-bath chloride gradient and decreased with a bath-to-lumen chloride gradient. The most likely explanation is that a bicarbonate-chloride exchanger is involved. The exact mechanism is now being investigated. In contrast to chloride, sodium was not required for bicarbonate secretion. The isolated perfused cortical collecting ducts secreted ammonia, as well as bicarbonate. The mechanism is nonionic diffusion dependent on a pH gradient caused by an acid pH disequilibrium in the lumen. The acid pH disequilibrium is caused by secretion of protons simultaneous with the secretion of bicarbonate. Its importance is that it provides for efficient excretion of ammonia even when the pH of the tubule fluid is not low.

Knepper has been studying role of urea in the concentrating mechanism and defining the mechanism of urinary concentration in the inner medulla. He is using perfusion of isolated tubules to define their transport parameters and mathematical modelling to define the mechanism by which the coordinate action of the tubules results in urinary concentration. The mathematical modelling studies have led to an expanded list of hypotheses for the mechanism of concentration. These hypotheses are currently being tested in studies of isolated nephron s egments and isolated papillary surface epithelium.

Necturus gallbladder epithelium.

The cellular aspects of epithelial transport can be studied by making measurements in individual cells. Since mammalian cells generally are very small, such direct studies are difficult and there are artifacts due to cell damage. Therefore, Spring and coworkers have been using the very large gallbladder cells of Necturus for this purpose. The gallbladder, like the renal proximal tubule, has a high transepithelial permeability to salt, and for this reason is called a "leaky" epithelium.

Leaky epithelia absorb large quantities of salt and water through their cells. Constancy of cell composition requires that the rate of entry of solutes and water into the cell across the apical cell membrane must be exactly balanced by exit across the basolateral cell membrane. Jensen and Spring have studied this regulation. They found that NaCl entry across the apical cell membrane was subject to feedback inhibition when cellular Na increased due to ouabain blockage of active Na transport out of the cells across their basal membranes. The feedback regulation did not occur, however, until after cell Na activity had increased threefold to 30 mM. Cell volume also increased for approximately 30 minutes following ouabain, then began to decrease towards normal. This late loss of cell water resulted from increased KCl exit across the basolateral cell membrane, followed by exit of water by osmosis. The KCl exit was inhibited quinidine, suggesting the involvement of calcium. Because the feedback inhibition of apical sodium permeability, which followed ouabain addition, required an increase in cell Na as high as 30 mM, it does not seem to be a likely candidate

for the close regulation of intracellular Na that normally is present. Instead it appears to be a response to extreme stress.

Regulation of epithelial cell volume in response to changes in bathing solution osmolality occurs very rapidly in Necturus gallbladder. The mechanism is a change in cell solute content, followed by osmosis of water. Spring and coworkers have been investigating the ionic mechanisms by which intracellular solute content is changed in response to osmotic stimuli. They are finding that epithelial cells constantly modulate the rate at which solutes enter and leave the cells in a way that controls both cell volume and ionic composition.

Larson and Spring studied the volume regulatory decrease that follows the initial swelling of cells exposed to hypotonic media. They found that volume regulatory decrease resulted from the loss of intracellular KCl across the basolateral cell membrane. The stoichiometry of this process was 3K to 2Cl. Volume regulatory decrease was sensitive to the bumetanide, which inhibits cotransport processes, but insensitive to inhibitors of ion exchange.

Foskett and Spring studied the activation of volume regulatory decrease. They found that the KCl exit depended on maintenance of calmodulin activity and on intact intracellular micro-filaments. Volume regulatory increase, on the other hand, was not dependent on calcium (or calmodulin) or on intact micro-filaments or other cytoskeletal elements.

Marsh and Spring showed that volume regulatory increase occurs as a result of activation of transport processes in the apical cell membrane, regardless of which side of the cells the osmotic challenge is presented to. Thus, the responses to osmotic stimuli are strictly polarized, i.e. volume regulatory increase occurs by the uptake of NaCl across the apical cell membrane while volume regulatory decrease occurs by loss of KCl across the basolateral cell membrane.

The mechanisms by which K and Cl leave epithelial cells during normal trans-epithelial transport are being investigated by Hermansson and Spring, utilizing a combination of optical, chemical and radioisotopic tracer methods. The work is still in progress, but up to this point they have found that most of the chloride flux occurs by ion exchange, while much of the K flux is by diffusion through conductive channels in the cell membranes.

Cell culture of epithelia.

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Handler, Burg and their colleagues have been using cultures of epithelial cells to overcome this difficulty.

Handler, Preston and Lang have been studying differentiation of renal epithelia in culture. A6 is a continuous line derived from the kidney of Xenopus laevis. Previously they found that the differentiation of A6 epithelia was greater when the cells were grown on permeable supports, such as millipore filters, that exposed the basal cell surface to medium, than when the cells were grown on plastic dishes, that did not. The investigators now find that a variety of

differentiated properties, including development of transepithelial resistance, adenylate cyclase responsiveness to vasopressin, and organization into a morphologically ordered epithelium all occur simultaneously. The pattern holds regardless of whether the cells are transferred to the filter from a plastic dish, in which case they begin relatively undifferentiated, or from a filter, in which case they are already differentiated. Differentiation was markedly accelerated by the addition of 100 nM dexamethasone, a synthetic adrenal corticosteroid. An assay for vasopressin receptors is now being developed in order to determine the time course of their expression.

Handler, Spiegel, Fishman, and Blumenthal are studying the polarity of distribution of complex lipids between the apical and basal plasma membranes of epithelia. They had proposed on the basis of earlier studies of renal epithelia in culture that only lipids in the inner leaflet of the plasma membrane could diffuse in the plane of the membrane past the tight junctions from the apical to the basolateral cell surface. They now find that GM1, the ganglioside that specifically binds cholera toxin, follows this pattern. They incorporated GM1 into the plasma membrane and followed its distribution by means of a fluorescent label. When GM1 was incorporated into the outer leaflet of the apical plasma membrane by applying it externally to the cells, it did not move to the basolateral membrane even though it entered the cells in endocytic vesicles. Endocytic vesicles are known to recombine with the plasma membranes continuously by fusion. Evidently, the ganglioside remained in the outer leaflet while in endocytic vesicles and the vesicles recombined only with the apical, and not the basal, plasma membranes. Studies using the specific binding of radioactive cholera toxin to GM1 in order to track its location confirmed the findings.

Sariban-Sohraby and Burg studied the sodium channels in the apical membranes of A6 cells. The channels are apparently similar to those in cortical collecting ducts and are important for the control of sodium reabsorption by that segment. In collaboration with Turner they prepared apical membrane vesicles from the cells and measured sodium uptake into the vesicles. Low concentrations of amiloride inhibited the sodium uptake, which is characteristic of these channels. When the cells were treated with aldosterone, the sodium uptake by their vesicles doubled. This is one of the sites at which the action of aldosterone is expressed. In collaboration with Johnson and Wiesmann they investigated the mechanism by which aldosterone increases the sodium flux. Previously, it had been shown that methylation of phospholipids and proteins was increased in cells after aldosterone. In the present studies the investigators found that S-adenosyl methionine added to the vesicles both caused methylation and caused the sodium flux to increase. The increase in flux was not additive to that caused by aldosterone and was prevented by inhibitors of methylation. Thus, methylation of membrane phospholipids or proteins is apparently involved in the increased sodium channel activity caused by aldosterone. The investigators also collaborated with Benos and Latorre in reconstituting sodium channels from the vesicles into planar lipid bilayers. With this preparation they were able to observe the electrical activity of single sodium channels and to characterize the properties of the channels. They measured single channel conductance, ionic selectivity, and the effect of amiloride. These studies represent a important advance towards defining the molecular characteristics of the channels.

Green and Burg have been developing lines of epithelial cells from various parts of the renal tubules. Although growth of differentiated primary cultures was relatively easy and was achieved several years ago, it has been difficult

to get the cells to grow continuously in lines. That problem has now been largely solved by discovery of better conditions for growing the cells. Important advances in this respect have been the use of denuded amnions as supports for the cells in early passages and addition of pituitary extract in place of serum in the medium. Lines of medullary thick ascending limbs were originated from single dissected segments and have now been in culture for up to two years. The have expressed transepithelial voltage and Tamm-Horsfall protein, which are specific for thick ascending limbs, in some, but not all passages. Current work is aimed at improving the maintenance of differentiation by cloning the cells and by defining conditions that will select for more differentiated cells.

Studies of artificial membranes.

Walter and Blumenthal have been using purified artificial systems to study the fusion of lipid membranes, a process that is important in renal cells. The components of cell plasma membranes are in a constant state of flux in which portions of the membranes are internalized into the cells and new membrane is constantly added by fusion of membrane vesicles from stores within the cells. This process serves many functions, including renewal of plasma membranes, regulation of the number and nature of transporters and receptors in them, and transport of molecules into and out of the cells. All of this occurs without loss of continuity of the plasma membranes, which remain effective barriers between the inside and outside of the cells. Thus, controlled membrane fusion is important for cell function. Nevertheless, the conditions required for it are not well defined. The investigators found that fusion of artificial lipid membranes was induced by addition of either of two small basic proteins, apocytochrome c or polylysine. They characterized the effects of protein binding, electrical charge, and pH. Both of the proteins promoted aggregation of membrane vesicles, followed by fusion, provided that at least 10 percent of the lipid was negatively charged. The maximum rate of fusion, for a given protein concentration, occurred at pH below 5.0, and the optimal protein concentration was found to be that which yielded a charge stoichiometry between lipids and protein close to 1:1. The investigators propose that electrostatic binding between two membranes causes them to come into close contact and increases the probability that fusion will occur in response to a trigger such as lowered pH. This model probably applies to the role of viral proteins in their fusion with cell membranes as well as to normal cellular membrane turnover, and endo- and exocytosis.

Metabolism associated with solute transport.

A large fraction of the metabolism of renal epithelial cells is utilized to produce energy for transepithelial transport. Balaban and his coworkers have been studying the pathways in intact cells by which metabolic pathways provide the energy for active solute transport. In order to accomplish this they have been developing and utilizing new non-invasive techniques for monitoring both the metabolic and transport pathways in intact cells. One of these new techniques is nuclear magnetic resonance (NMR) which can monitor the concentration and kinetics of intracellular metabolites in intact tissues.

Balaban, Kantor, and Ferretti have used two dimensional and saturation transfer NMR to study the rates of enzymatic reactions involved in metabolism. As reported last year, they began by characterizing in purified systems the reactions

catalyzed by creatine kinase, ATPase, and adenylate kinase. Then, they extended the method to study intact organs in vivo. They now have found that, although saturation transfer and two dimensional NMR can both be used to measure the rates of the metabolic reactions, there are subtle differences between the methods that yield important additional information. The signal from a particular type of molecule in the two dimensional technique is directly proportional to the amount of that compound. Therefore, small pools and those whose signal is at the same frequency as larger pools are not detected. Those pools can be traced, however, by saturation transfer. The difference allows the distinction of the different pools of metabolites and enzymes within transporting tissues. The investigators are now applying the techniques to tissues in vivo and to epithelial cells in culture.

Kantor, Briggs and Balaban developed a new NMR probe, the catheter coil, which permits the acquisition of NMR information from internal organs such as the heart and the alimentary canal. Previous information about metabolism in these tissues came largely from in vitro studies. The investigators used the new coils for cardiac catheterization of dogs and also passed them into the stomachs of dogs. They found that the metabolic control mechanisms differed in vivo compared to in vitro, especially in the heart which is highly active metabolically. Because techniques already exist for introduction of such catheters into patients, it is likely that the technique can be used clinically to study the metabolic defects in diseased organs.

Balaban and Knepper previously demonstrated by 14N NMR that high concentrations of trimethylamines exist in the kidney medulla. These compounds probably protect the renal cells from the high osmolality and high urea concentration in the renal papilla, but details of the protection and the consequences of its failure remain to be worked out. NMR was not sensitive enough to resolve the individual trimethylamines in experiments using practical amounts of tissue. The exact chemical identity of the trimethylamines and the control of their concentration is now being determined in collaboration with Bagnasco. The investigators have set up chemical methods for analyzing some of the trimethylamines (choline, phosphoryl choline, and phosphoryl glycerol choline), and are developing methods for others (such as betaine). Up to now, the principal trimethylamine found in the rat papilla was phosphoryl glycerol choline. In collaboration with Green and Burg they found a high concentration of phosphoryl glycerol choline also in cells of a continuous culture from the rabbit papilla. They are now determining effects of water deprivation and loading on the trimethylamines in rat papillas and of medium hyperosmolality and urea concentration in the cultured rabbit papillary cells.

Balaban and Kurtz have developed a microspectrophotometric system which permits them to topologically monitor intracellular pH. Using this device they are investigating intracellular pH regulation and cellular metabolic acidosis in cultured epithelial cells and isolated perfused tubules.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01217-09 KE

PERIOD COVERED

October 1, 1983 to March 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Epithelial Fluid Transport and Morphology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

P.I.	Kenneth R. Spring	Physiologist	LKEM, NHLBI
Others:	Mikael Larson	Visiting Fellow	LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.75	.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism of volume regulatory decrease in osmotically swollen epithelial cells was studied by quantitative light microscopy and ion sensitive microelectrodes. Volume regulation was shown to occur because of the activation of a quiescent transport system for KCl.

623

Objectives

The primary goal of this investigation is the elucidation of the mechanism of volume regulation by epithelial cells. We wish to determine the site and mechanism of the volume regulatory decrease which follows osmotic swelling of Necturus gallbladder epithelial cells.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties and variation in solution composition. The chamber is placed on the stage of an inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations. Ion sensitive microelectrodes were constructed as previously described and used to measure the intracellular activities of K and Cl.

Major Findings

Volume regulatory decrease occurs because the cells lose KCl in response to osmotic swelling. The KCl is transported across the basolateral cell membrane by a coupled process involving the neutral movement of both ions. This exit process was inhibited by bumetanide, a diuretic, and by histrionicatoxin, a neurotoxin. Manipulation of the chemical gradients for K and Cl across the basolateral cell membrane altered the rate of volume regulation in hypotonic solution. Measurement of the intracellular activities of K and Cl with the solutions utilized for the volume regulation studies allowed us to calculate the stoichiometry of the KCl cotransport process. The exit of KCl occurred with a transport ratio of 3K to 2Cl ions.

Proposed Course

Project completed.

Publications

Larson, M. and K.R. Spring: Volume regulation in Necturus gallbladder: Basolateral KCL exit. J. Membr. Biol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01224-07 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of sodium and potassium transport by the nephron

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	M.A. Knepper	Med. Staff Fellow	LKEM, NHLBI
Others:	Kimio Tomita	Visiting Fellow	LC, NHLBI
	J.J. Pisano	Res. Chemist	LC, NHLBI
	Maurice B. Burg	Investigator	LKEM, NHLBI

COOPERATING UNITS (if any)

Physiological Chemistry Section, Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effects of mineralocorticoids, arginine vasopressin, and bradykinin on isolated perfused cortical collecting ducts from rats are being investigated. Chronic deoxycorticosterone treatment of the rats increases sodium absorption and potassium secretion by the collecting ducts in vitro. Addition of arginine vasopressin to the bath in vitro increases both the sodium and potassium transport above the deoxycorticosterone-stimulated levels. Addition of bradykinin to the bath in vitro inhibited the sodium absorption, but did not significantly affect potassium transport.

625

Objectives

The chief long term goal of this project is to determine the mechanisms of control of sodium and potassium excretion by the kidney. The current emphasis is on identification of the hormonal signals that may affect electrolyte transport in collecting duct segments.

Methods

Cortical collecting ducts are dissected from rat kidneys and perfused in vitro. Sodium, potassium, and inulin concentrations are measured in the perfusion fluid, the bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. Potential difference is measured across the epithelium. Vasopressin (AVP) and/or bradykinin (BK) in physiological concentrations are placed in the bath or perfusate after control measurements are made to determine the effects of the agents on the measured variables. Some of the animals are pretreated with deoxycorticosterone (DOC) to assess the effect of long term exposure to increased levels of adrenal corticosteroids with mineralocorticoid activity.

Results

The tubules were perfused at 1.5-2.5 nl/min/mm. The following are mean values for the experimental series completed thus far:

Animal Treatment	In vitro Hormone* (in bath)	Flux (peq/min/mm)		Potential Difference, (mv)
		Na	K	
None	None	0.2	0.1	-0.8
DOC	None	13.8	-2.1	-3.6
	BK	7.2&	-1.5	-2.1
DOC	None	12.5	-5.1	-10.7
	AVP	58.0&	-16.6&	-26.6&
	None	27.7	-7.3	-13.6
DOC	AVP	51.0	-12.0	-24.5
	AVP+BK	31.2&	-9.7	-22.1
	AVP	44.8	-6.2	-21.6

* AVP, 10^{-10} M. BK, 10^{-9} M.
& Significantly different from first value in set.

Thus, with no treatment of the animals and no hormone in the bath there was no transport of sodium or potassium, and the transepithelial potential difference was virtually zero. Deoxycorticosterone treatment of the rats resulted in measurable absorption of sodium and secretion of potassium in the isolated

tubules, but the values are considerably lower than observed in cortical collecting ducts from deoxycorticosterone treated rabbits. Vasopressin added to the bath however markedly increased both the sodium absorption and the potassium secretion, while also increasing the transepithelial potential difference. This contrasts with prior results in the rabbit where vasopressin markedly inhibited the sodium flux and voltage of isolated cortical collecting ducts. Bradykinin substantially inhibited the sodium flux without a significant effect on potassium flux or potential difference.

Significance

The cortical collecting duct is an important site of Na reabsorption and potassium secretion. It is a major site of control of urinary Na and K excretion. Evidence from other types of studies suggests that mineralocorticoids, vasopressin and the kallikrein-kinin system play in important role in the regulation of renal electrolyte excretion. These studies indicate that all three exert important effects on electrolyte transport by the cortical collecting duct of the rat.

Proposed Course

Further studies are proposed to examine the effect of these agents on chloride and bicarbonate transport by the cortical collecting duct of the rat. The possible role of prostaglandins in the bradykinin induced inhibition of sodium reabsorption will be examined.

Publications

Proud, D., M.A. Knepper, and J.J. Pisano. Distribution of immunoreactive kallikrein along the rat nephron. Am. J. Physiol. 244:F510-F515, 1983.

Knepper, M., and M. Burg. Organization of nephron function. Am. J. Physiol. 244:F579-F589, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01237-06 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal control of transport in kidney epithelia in culture.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Michael A. Lang	Expert	LDN, NICHHD
	Agnes Scott Preston	Research Chemist	LKEM, NHLBI
Others:	Jacqueline Muller	Senior Investigator	DBB, NCDB
	Harold Gainer	Senior Investigator	LDN, NICHHD
	Joseph S. Handler	Senior Investigator	LKEM, NHLBI

COOPERATING UNITS (if any)

Laboratory of Developmental Neurobiology, NICHHD and Division of Biochemistry and Biophysics, National Center for Drugs and Biologics

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.6	2.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The response to hormones is studied in an epithelium as it forms in culture. Epithelia formed by A6 cells, derived from kidney of Xenopus laevis, do not respond to vasopressin when grown on a petri dish. When grown on a millipore filter bottom cup, the epithelia respond to vasopressin. After seeding on a filter, the responsiveness of each subunit of adenylate cyclase was evaluated over a period of two weeks. cAMP accumulation was measured in response to stimulation of hormone receptor (by vasopressin), G nucleotide binding regulatory subunit (by cholera toxin), and catalytic subunit of adenylate cyclase (by forskolin). Evaluation was also performed by assays of adenylate cyclase activity in membrane rich fractions prepared from homogenates of the epithelia. The activity of each subunit appears to develop with a different time course. In addition, there is a close correlation between the development of vasopressin sensitive adenylate cyclase, transepithelial electrical resistance, and formation of a morphologically ordered epithelium.

6 25

Objectives

The purpose of this study is to examine the cellular factors involved in the hormonal regulation of epithelial sodium transport. Current work is focused on the stimulation of sodium transport in epithelia formed in culture by A6 cells, a continuous line derived from the kidney of Xenopus laevis. We have described the transport properties of the epithelium and its responses to hormones in previous reports. We have also identified culture conditions that lead to epithelia that lack responsiveness to vasopressin, as well as conditions that lead to a marked stimulation of adenylate cyclase by vasopressin and subsequent stimulation of sodium transport. The objective is to identify the cellular changes that occur as the epithelium becomes responsive to vasopressin. This will lead to a greater understanding of differentiation of epithelial function as well as a better understanding of hormonal control of sodium transport.

Methods

See previous annual reports for methods of cell culture, of measuring sodium transport, cAMP, and adenylate cyclase activity. Epithelia are fixed with glutaraldehyde for preparation of thick sections for staining and light microscopy and for thin sections for transmission electron microscopy. Receptors for vasopressin are studied by measuring the specific binding of radiolabelled vasopressin to whole epithelia, single cell suspensions prepared from epithelia by chelation of calcium, and crude membrane preparations from disrupted cells.

Major Findings

There is a concomitant development of adenylate cyclase responsiveness to vasopressin, transepithelial electrical resistance, and morphologic organization into an ordered epithelium. The parallelism occurs in epithelia subcultured onto filters following growth on plastic petri dishes (our standard preparation), and in epithelia subcultured from filters, in which case the cells have differentiated fully before they are subcultured.

Specific binding of radiolabelled vasopressin to intact epithelia and to intact single cells chelated from epithelia have not yielded sufficient signal (specific binding) compared to noise (non-specific binding) in the time in which binding would occur related to the adenylate cyclase response to the hormone. Preliminary experiments with membrane fractions prepared from disrupted cells have minimally satisfactory specific binding, indicating that we will be able to assess receptor binding. Over longer periods of exposure to vasopressin, single cells do manifest "specific binding" of the hormone. This is seen in cells prepared from epithelia that have an adenylate cyclase response to vasopressin (growth on a filter) and from cells prepared from epithelia that do not have an adenylate cyclase response to vasopressin (growth on a petri dish). This specific binding may be specific uptake (endocytosis). It is highly temperature sensitive and is linear for up to six hours. It is pH sensitive, showing maximal uptake at pH 6.5. The biologic significance of this specific uptake remains to be determined.

Finally, we have discovered that adrenal steroid hormones have a profound effect on the differentiation of A6 cells. We have previously described a typical mineralocorticoid effect of aldosterone on sodium transport by A6 epithelia. We

now find that when mature epithelia grown on a filter are incubated with adrenal steroid hormones, vasopressin sensitive adenylate cyclase doubles. The time course is typical for a steroid elicited response. Dexamethasone is more potent than aldosterone. The effect is on maximal vasopressin stimulated activity rather than on the concentration of vasopressin that elicits half maximal responsiveness, i.e.- an increase in Vmax rather than affinity for vasopressin. The addition of dexamethasone to cells when they are first seeded on filters accelerates markedly the development of transepithelial resistance as well as vasopressin sensitive adenylate cyclase. Effects on morphology of the epithelium remain to be evaluated. The standard growth medium contains 10% fetal bovine serum. When dexamethasone is substituted for fetal bovine serum immediately after cells are seeded onto filters, epithelia differentiate as completely and more rapidly than they do in the standard growth medium containing serum.

Proposed Course

The assay for vasopressin receptors related to the adenylate cyclase response will be developed further so that receptors for vasopressin can be assayed in epithelia grown under conditions we have found alter the response to the hormone.

The specific uptake of vasopressin by intact single cells over long periods will be evaluated further to see whether it is related to the adenylate cyclase response.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01238-02 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ammonia and lactic acid production by individual segments of the rat nephron

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Serena Bagnasco	Visiting Associate	LKEM, NHLBI
Others:	David Good	Staff Fellow	LKEM, NHLBI
	Robert Balaban	Sr. Staff Fellow	LKEM, NHLBI
	Maurice Burg	Investigator	LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.9	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Lactate production has been measured in isolated segments of rat nephron. Production rates were determined under aerobic and anaerobic conditions. Rates obtained when oxidative metabolism was inhibited by antimycin A were used to evaluate the glycolytic capacity of each segment during anoxia. In the next series of experiments ammonia production will be measured in individual nephron segments of potassium depleted and potassium loaded rats. The aim is to determine in which sites of the nephron ammoniagenesis is altered in response to in vivo changes of potassium balance.

631

Objectives

The direct determination of lactate production in isolated segments of rat nephron was aimed to evaluate glycolytic metabolism among different nephron segments. Potassium depletion and potassium loading are known to alter renal ammoniagenesis. Ammonia production will be measured in individual nephron segments of potassium depleted and potassium loaded rats to determine which segments modify their production in response to altered potassium homeostasis.

Methods

Tubule segments dissected from collagenase treated rat kidneys are incubated under oil in microliter fluid droplets of known composition. Nanoliter samples are removed from the droplets at 10 minute intervals and assayed for lactate or ammonia. Lactate is measured using lactate dehydrogenase in nanoliter volumes. Ammonia is measured using glutamate dehydrogenase. Both reactions involve changes in NADH concentration, which are measured by microfluorimetry and used to quantitate sample lactate or ammonia content.

Major Findings

All segments tested except the proximal tubule were capable of lactate production under aerobic and anaerobic conditions. Impairing oxidative metabolism with antimycin A caused production rates to increase significantly in all distal segments. The high increments observed in medullary thick ascending limb, cortical and outer medullary collecting duct suggest that during anoxia a significant amount of ATP may be generated by glycolysis in these segments.

Significance

When oxidative metabolism is impaired, as during anoxia, glycolysis is the major alternative pathway for ATP generation. Different capabilities in performing glycolysis among different nephron segments may in part explain the observation that renal anoxia affects some portions of the nephron more than others. The factors that may regulate renal ammonia production in response to changes in acid-base and potassium homeostasis are still undefined. Also, the specific sites of the nephron in which ammonia production is altered during potassium loading and potassium depletion remain to be determined. Measuring ammonia production in individual nephron segments from potassium depleted and potassium loaded rats may provide important information in this regard.

Publications

Bagnasco, S., M. Burg, R. Balaban, D. Good: Lactic acid production by nephron segments of the rat. AAP/ASCI/AFCR Annual Meeting (abstract), May 4-7, 1984, Washington, D.C.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01244-05 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of glucose transport by cultured kidney epithelial cells.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. Moran	Guest Worker AFFRI, LKEM, NHLBI
Others:	R. J. Turner	Visiting Associate LKEM, NHLBI
	M. Hagan	Dept. of Exp. Hematology, AFRRI
	J.S. Handler	Investigator LKEM, NHLBI

COOPERATING UNITS *(if any)*

Department of Physiology and Department of Exprimental Hematology, AFRRI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Sugar transport is studied in a cultured epithelium formed by cells derived from pig kidney. Transport is assessed by measuring sodium dependent uptake of analogs of glucose into the epithelium as well as into apical membrane vesicles prepared from the epithelium. The number of apical membrane sodium-coupled glucose transporters is evaluated by measuring the specific binding of radiolabelled phlorizin. Growth of the epithelia in a medium containing high concentrations of glucose results in fewer apical memrane glucose transporters compared to growth in normal concentrations of glucose. When cell division is impaired following ionizing radiation, the increase in the number of apical membrane glucose transporters usually elicited by incubation in a low glucose medium is impaired. In contrast, ionizing radiation does not impair the loss of apical membrane glucose transporters seen when cells grown in a low glucose medium are switched to a medium containing a high concentration of glucose.

635

Objective

We and others have demonstrated that epithelia formed in culture by cells of the kidney-derived continuous line designated LLC-PK1 manifest sugar transport like that of the proximal tubule of mammalian kidney. The special feature of this transport system is the sodium-coupled glucose transporter in the apical membrane. Factors that affect the development of the transporter and its function will be examined to gain further understanding of the regulation of expression of differentiated epithelial functions in cultured epithelia as well as further understanding of the glucose transport system in the kidney.

Methods

See previous reports for methods of growing epithelia formed by LLC-PK1 cells, for assessing apical membrane sodium-coupled glucose transport, and for other assays. Cell division is assayed by measuring the incorporation of radiolabelled thymidine into DNA. The standard method, used for studying fibroblasts, was modified for these studies with epithelia. The modification consists of a chase of cold thymidine following incorporation of radiolabelled thymidine. The modification results in considerable increase in sensitivity for detecting the incorporation of radiolabelled thymidine into DNA. Cells are irradiated at the cobalt-60 gamma radiation facility at AFRRI.

Major Findings

See previous reports for information regarding the effect of the concentration of glucose in the growth medium on the number of sodium-coupled hexose transporters in the apical membrane of LLC-PK1 epithelia.

Since the response to a change in the concentration of glucose in the growth medium is slow (the earliest change is detectable after 24-48 hours), the role of cell proliferation was examined. After cultures are confluent and cell number per unit area is stable, there is still thymidine incorporation into DNA, indicating continual cell division and cell death. Under those conditions, cessation of cell division that results from irradiation impairs the increase in glucose transport that normally results when LLC-PK1 epithelia are shifted to a growth medium containing a low concentration of glucose from a medium containing a high concentration of glucose. In contrast, irradiation that halts cell division does not impair the decrease in the rate of glucose transport that normally occurs when cells grown in a medium containing a low concentration of glucose are shifted to a medium containing a high concentration of glucose.

Proposed Course

Future studies will be conducted by A. Moran at AFRRI.

Publications

Moran, A., R.J. Turner and J.S. Handler. Regulation of sodium-coupled transport by glucose in a cultured epithelium. J. Biol. Chem. 258:15087-15090, 1983

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01246-04 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Primary and continuous culture of epithelial kidney cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Nordica Green	Chemist	LKEM, NHLBI
Other:	Maurice B. Burg	Chief,	LKEM, NHLBI
	Hayden Coon		NCI
	Timothy Triche		NCI
	John Hoyer	Children's Hospital, Philadelphia, PA	

COOPERATING UNITS *(if any)*

Children's Hospital, Philadelphia, PA

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose is to establish continuous lines of renal epithelial cells that express the transport properties of the tubule segments from which they originated. Continuous lines have been developed from papillary pelvic lining epithelium and medullary thick ascending limb. These tissue culture epithelia express some differentiation, but this differs between lines and between passages of a particular line.

6.35

Objectives

The long term goal of the studies is to develop continuous lines of renal tubule epithelial cells that continue to express the differentiated transport properties of the nephron segments from which they originate in order to study those properties in vitro.

Methods

Fragments of identified single tubule segments or of papillary pelvic epithelium were dissected and placed in culture. Suitable media and substrata were selected empirically. Use of denuded sheep amnion or collagen coated dishes as supporting structures and supplementation of the medium with hormones, including pituitary extract, were particularly useful.

Major findings

A number of epithelial cell lines are now in culture through multiple passages. They include: GRB-PAP1 from the papillary pelvic epithelial lining is in passage 38 after 6 years of culture. Papillary cells in vivo are specially adapted to withstand the high osmolality and urea concentration in that region. The adaptations are poorly understood, but are believed to include high concentrations of trimethylamines in the cells. Generally, cells in culture do not withstand anisotonic media, but a strain of GRB-PAP1 has now been growing in hypertonic NaCl for more than one year. These cells contain a high concentration of phosphoryl glycerol choline and are being used to study its role in adaptation to hypertonicity.

GRB-MAL1 from medullary thick ascending limb is in passage 20 after two years of culture. Like the other epithelial cell lines described here it maintains excellent morphological differentiation with polarization of apical and basolateral membranes and well preserved zona occludens on electron microscopy. It expresses Tamm-Horsfall protein (detected by specific immunofluorescence), which is limited to thick ascending limb in vivo, and therefore is indicative of specific differentiation of these cells.

GRB-MAL2 in passage 10 after one year had a transepithelial voltage characteristic of thick ascending limb in some early passages, but this disappeared when the cells became visibly heterogeneous. Methods have now been developed for clonal growth of the MAL cells and some clones are now being examined for differentiation.

Lines are being grown from suspensions of renal cortical cells enriched with proximal tubules. Proximal tubule cells are selected by controlling the type of nutrient. Proximal tubules are the only segments in the kidney that are gluconeogenic. Also, they have specialized transport systems (e.g. sodium-dependent glucose and amino acid transporters) and brush border enzymes (e.g. polysaccharidases and oligopeptidases). Growth has been accomplished in glucose-free media, containing trehalose, maltose, or alpha-ketoglutarate as the principal nutrients.

Proposed course

(1) To attempt to start lines from dissected fragments of other segments in addition to the thick ascending limbs.

(2) To attempt to achieve more consistent differentiation by cloning and by use of media that contain special nutrients. Limitation of the nutrients is intended to force selection of cells with enzymes and transporters specific for those nutrients.

(3) To attempt to immortalize the cells by transfecting them with the appropriate oncogenes. Experiments with v-myc in the proximal tubule cells are underway.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01247-04 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Urea transport and the urinary concentrating mechanism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Mark Knepper	Med. Staff Fellow LKEM, NHLBI
Others:	J.L. Stephenson	Investigator TBS, NHLBI
	Jeff Sands	Med. Staff Fellow LKEM, NHLBI

COOPERATING UNITS *(if any)*

Theoretical Biology Section, NHLBI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of isolated renal tubules and mathematical modelling studies are being carried out to investigate the role of urea in the concentrating mechanism and to define the mechanism of concentration in the inner medulla. Mathematical modelling studies have led to an expanded list of hypotheses for the mechanism of concentration in the inner medulla. These hypotheses are currently being tested in studies of isolated nephron segments and isolated papillary surface epithelium.

63

Objectives

The chief long term goal is to define the role that urea plays in the renal concentrating mechanism. The current emphasis is on the mechanism of concentration of the inner medullary interstitium which is the major unresolved issue in the concentrating mechanism field.

Methods

Microdissection techniques are used to isolate the individual epithelial components of the kidney. Isolated nephron segments are perfused in vitro. Ultramicro methods for the measurement of urea, chloride, inulin, and osmolality are used in isolated perfused tubule studies to characterize the transport properties of each nephron component of the kidney. Mathematical modelling techniques are used to analyse the data from the point of view of their overall significance to the aggregate function of the kidney.

Major Findings

In collaboration with Dr. John Stephenson, we have generalized Stephenson's previous analysis of the mass balance requirements for concentration in the renal medulla. The major conclusion is that in principle the urine can be concentrated by diluting any ascending stream or by concentrating any descending stream in the medulla with respect to the interstitium at the same level. The consequence of this is that the "single effect" for concentration in the inner medulla could exist in any of the parallel elements. Descending streams in the medulla include the thin descending limb, the inner medullary collecting duct, the descending vasa recta, and the peri-papillary pelvic space during part of the pelvic contraction cycle. Ascending streams include the thin ascending limb, the ascending vasa recta, and the peri-papillary pelvic space during part of the pelvic contraction cycle. This analysis provides a theoretical framework on which to base a systematic experimental search for the single effect in the inner medulla of the mammalian kidney.

In collaboration with Drs. Saidel and Chandhoke, we have carried out simulations of the renal concentrating mechanism using a detailed mathematical model of urea, salt and water transport in the kidney. We have specifically evaluated the role of active NaCl absorption from the inner medullary collecting duct in inner medullary function. Although, this transport process augments the urinary concentrating capacity, the simulations show that it is unlikely to play a central role in the inner medullary concentrating mechanism, but rather is chiefly important for conservation of NaCl.

Significance

Included in previous section.

Proposed Course

We have begun to isolate and study thin ascending and descending limbs from the inner medulla. We plan to characterize the transport properties of these segments. We are also developing a method for the isolation and study of the

papillary surface epithelium which may play an important role in the inner medullary concentrating mechanism.

Publications

Knepper, M.A. Urea transport in nephron segments from medullary rays of rabbits. Am. J. Physiol. 244:F622-F627, 1983.

Knepper, M.A. Urea transport in isolated thick ascending limbs and collecting ducts from rats. Am. J. Physiol. 245:F634-F639, 1983.

Balaban, R.S., and M.A. Knepper. Nitrogen-14 nuclear magnetic resonance spectroscopy of mammalian tissues. Am. J. Physiol. 245:C439-C444, 1983.

Knepper, M.A., and J.L. Stephenson. Urinary concentration and dilution. In: Physiology of Membrane Disorders (2nd edition), edited by T.E. Andreoli, J.F. Hoffman, D.D. Fanestil, and S.G. Schultz. New York: Plenum, 1984 (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01250-04 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Acidification and bicarbonate transport by renal tubules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	M.A. Knepper	Med. Staff Fellow	LKEM, NHLBI
Others:	David Good	Staff Fellow	LKEM, NHLBI
	Juan Garcia-Austt	Guest Worker	LKEM, NHLBI
	Robert Star	Med. Staff Fellow	LKEM, NHLBI
	Maurice B. Burg	Investigator	LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3	3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of ammonia and bicarbonate transport are being conducted in isolated, perfused thick ascending limbs and cortical collecting ducts from rats and rabbits. Ammonia is absorbed by thick ascending limbs. This probably results from passive NH_4^+ transport driven by the lumen positive transepithelial electrical potential difference. Bicarbonate is actively absorbed by thick ascending limbs of rats. This results from secondary active secretion of protons, proabably mediated by sodium-proton exchange across the apical membrane. Active bicarbonate secretion in the cortical collecting duct is stimulated by treatment of the experimental animals with deoxycorticosterone. The stimulation of bicarbonate secretion is prevented by acid loading the animals. Bicarbonate secretion is dependent on the presence of chloride in the perfusate and bath. The rate of bicarbonate secretion is increased by a lumen-to-bath chloride gradient and is decreased by a bath-to-lumen chloride gradient. Bicarbonate secretion is not dependent on the presence of sodium in the solutions. The isolated perfused cortical collecting duct spontaneously secretes ammonia. This has been shown to be dependent on an acid pH disequilibrium in the lumen and probably involves diffusional entry of NH_3.

641

Objectives

The chief long term goal of this project is to determine the mechanisms of net acid and buffer transport by the kidney and how these transport processes are controlled. The current emphasis is on ammonia and bicarbonate transport in the thick ascending limb and cortical collecting duct.

Methods

Cortical collecting ducts or thick ascending limbs are dissected from rat or rabbit kidneys and perfused in vitro. Total carbon dioxide, total ammonia, chloride, and/or inulin concentrations are measured in the perfusion fluid, the bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. Potential difference is measured across the epithelium.

Results

1. Ammonia and bicarbonate transport in the thick ascending limb of rat. Ammonia is absorbed against a concentration gradient. Bicarbonate is absorbed simultaneously. Consequently, the total ammonia transport is occurring against a concentration gradient for NH_3. Most likely, the ammonia transport results from passive absorption of NH_4^+ driven by the lumen positive voltage. The bicarbonate transport is active. It is inhibited by acetazolamide, amiloride, sodium removal, and bath potassium removal. It is stimulated by luminal furosemide. Most likely, the bicarbonate transport results from the secondary active transport of protons into the lumen via a Na-H exchanger.

2. Ammonia and bicarbonate transport by the cortical collecting duct of the rabbit. Chronic deoxycorticosterone administration to the rabbits stimulates a high rate of bicarbonate secretion by the cortical collecting duct in vitro. Simultanous total ammonia secretion was demonstrated. Addition of ouabain to the bath does not significantly affect the ammonia secretion or bicarbonate secretion. Addition of the enzyme carbonic anhydrase to the luminal perfusate converted the net ammonia secretion to absorption, but did not alter the bicarbonate secretion. Thus, the ammonia secretion is dependent on a luminal pH disequilibrium and probably involves NH_3 diffusion into the lumen. The bicarbonate secretion is inhibited by luminal chloride replacement and stimulated by bath chloride replacement. The bicarbonate secretion is eliminated by chloride replacement on both sides of the epithelium, but not by complete sodium replacement. The rate of bicarbonate secretion does not correlate with transepithelial potential difference. Most likely, the bicarbonate secretion involves neutral bicarbonate-chloride exchange. The metabolic energy transducer ("pump") for the process is as yet unidentified.

Measurements of unidirectional (bath-to-lumen or lumen-to-bath) bicarbonate fluxes revealed that replacement of chloride in the lumen by sulfate markedly inhibited the bicarbonate secretory flux, and had little or no effect on the bicarbonate absorptive flux. Luminal sulfate also made the transepithelial potential difference more negative by 8-15 mv, a change too small to affect substantially the rate of proton secretion.

Acid loading the rabbits with oral NH_4Cl and rat chow prevents the bicarbonate secretory response of the cortical collecting ducts to deoxycorticosterone and also prevents the metabolic alkalosis induced by deoxycorticosterone. The stimulation of bicarbonate secretion may therefore be a response to the metabolic alkalosis rather than to the steroid. Measurements of unidirectional (bath-to-lumen or lumen-to-bath) bicarbonate fluxes revealed that the acid-loading markedly inhibited the bicarbonate secretory flux and did not substantially stimulate the bicarbonate absorptive flux.

3. Ammonia and bicarbonate transport by cortical collecting ducts of the rat. Deoxycorticosterone treatment of the rats results in a high rate of bicarbonate secretion in their cortical collecting ducts as occurs in rabbits. This response is prevented by acid loading. Addition of 4 mM total ammonia to the bath and perfusate solutions converted the net bicarbonate secretion to absorption. This contrasts with prior results in the rabbit where the presence of ammonia did not inhibit the bicarbonate secretion.

Significance

These studies demonstrate the the thick ascending limb of the rat is an important site of ammonia absorption from the loop of Henle, and an important site of sodium-dependent acidification in the distal nephron. The studies demonstrate direct transport of the NH_4^+ form of ammonia, and therefore we conclude that nonionic diffusion is not the sole mechanism of total ammonia transport along the nephron.

We have shown that the cortical collecting duct is an a site of bicarbonate secretion at rates that are likely to be of great importance with regard to the control of net acid excretion particularly in metabolic alkalosis or when dietary intake of alkali is high. Bicarbonate absorption in this segment can be unmasked by acid loading or creation of metabolic acidosis. However, the rate of bicarbonate absorption is low in this segment when compared to that in the outer medullary collecting ducts. Bicarbonate absorption in the cortical collecting duct results from active proton secretion. Proton secretion in this segment may be important primarily for its ability to lower the pH in the lumen by creating an acid pH disequilibrium. This will increase the driving force for ammonia secretion by nonionic diffusion and therefore enhance the rate of buffer delivery.

Proposed Course

Further studies are proposed to examine in more detail the mechanism of bicarbonate secretion in the cortical collecting duct. Attempts will be made to identify factors that control the rate of bicarbonate secretion. The mechanism of ammonia transport across the basolateral membrane of the cortical collecting duct cells will be examined. We will begin to examine ammonia and bicarbonate transport at other sites along the nephron, viz. the proximal straight tubule, the thin limbs and the medullary collecting duct. Mathematical modelling studies will be done to aid in designing experiments, in interpreting the data, and in integrating the results into an overall view of acid-base transport in the kidney.

Publications

Good, D.W., Knepper, M.A., and M.B. Burg. Ammonia and bicarbonate transport by thick ascending limbs of rat kidney. Am. J. Physiol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01251-04 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The application of nuclear magnetic resonance to the study of cellular physiology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Robert S. Balaban	Sr. Staff Fellow LKEM, NHLBI
Others:	Howard L. Kantor	Med. Staff Fellow LKEM, NHLBI
	Richard Briggs	Assistant Professor HMS, U. Pa.

COOPERATING UNITS (if any)

Department of Radiology, Hershey Medical College, Hershey, PA

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Radiofrequency coils have been developed that can be used as intravenous or arterial or alimentary catheters. These catheters were placed in the right and left ventricle and at various locations along the alimentary canal of anesthetized dogs under fluoroscopic observation. 31P and 1H NMR signals were then obtained from these coils. 31P NMR signals were detected from creatine phosphate, inorganic phosphate and adenosine triphosphate with adequate signal to noise within five minutes. This technique will be extremely useful in investigating the in vivo energy metabolism of internal organs with minimal surgical intervention.

PHS 6040 (Rev 1/84)

GPO 904-917

Objectives

The use of ^{31}P NMR to study the energy metabolism of intact tissues in vivo has generally been limited to external tissues such as skeletal muscles or those tissues which can be surgically exposed. In this study we investigated the use of intravenous catheter radiofrequency coils to detect signals from internal organs of anesthetized dogs.

Methods

Catheter radiofrequency coils for intravenous and arterial studies were made from a two-turn copper wire coil formed in an ellipzoid shape approximately 20 cm long and 0.5 cm wide. The coil was coated with a lowloss coating and tuned with a microchip capacitor to 32 mHz, the detecting frequency for ^{31}P. This coil was attached to a non-magnetic coaxial cable of 2 mm in diameter and 27 cm in length. Studies on the alimentary canal were made with circular coils 2.5 cm in diameter. The catheter coils were then inserted in an anesthetized dog under fluoroscopic observation. The experiments were performed in an Oxford instrument wide bore spectrometer detecting ^{31}P at 32 mHz. The field was shimmed using the ^{1}H signal from the water around the coil.

Major Findings

The catheter coil design we used showed little loss in sensitivity when placed within the blood stream of the animal and could be easily localized under fluoroscopy. The coil position remained stable for up to five hours. ^{31}P NMR spectra of creatine phosphate and ATP were obtained from the in vivo dog. Five minutes of data collection was sufficient to produce >30/1 signal to noise for CrP in the right ventricle studies. Preliminary studies on the right ventricle have demonstrated that very large increases (two-fold) in heart work output upon pharmacological stimulation with dobutamine or ouabain had little or no effect on the steady state concentration of high energy phosphate compounds. This result is in contrast to in vitro studies on perfused hearts where very small increased in work output have resulted in large changes in CrP and Pi levels. Presumably the heart is much more capable of maintaining its high energy phosphates in vivo. The mechanisms of this regulation is now under investigation. ^{31}P and ^{1}H NMR spectra were also collected from the fundic region of the stomach and small intestine of the anesthetized dog demonstrating the utility of the procedure for the alimentary canal.

Proposed Course

To further investigate the relationships between cardiac work output and high energy phosphate compounds in the right and left ventricles in vivo. Cardiac pacing and ^{31}P NMR aquisitions gated to the cardiac cycle will be used instead of the more complicated pharmacologic alterations of cardiac work output.

Publications

Kantor, H.L., R.W. Briggs and R.S. Balaban: In vivo ^{31}P NMR measurements in canine heart using a catheter-coil. Circulation Res. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01261-02 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intracellular pH regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	R.S. Balaban	Sr. Staff Fellow	LKEM, NHLBI
Others:	Ira Kurtz	Guest Worker	LKEM, NHLBI
	Paul Smith	Visiting Scientist	DRS, NIH
	Ronald Lynch	Guest Worker	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Division of Research Services

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.7	1.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism of intracellular pH regulation in isolated perfused renal tubules and epithelial cells in culture is under investigation. We are using a unique fluorescent pH probe, 1,4 Dihydroxyphallonitrile, which has both a fluorescent acid (blue) and base (green) form. This probe in a cell used in combination with a rapid scanning microfluorometer on a microscope permits the topological assessment of intracellular pH. Currently we are studying the mechanisms of intracellular proton buffering mechanisms with regards to external and intrinsic acid loads.

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Objectives

(1) To build a microspectrophotometer capable of topologically monitoring intracellular dyes and intrinsic cellular fluorescence. (2) Develop appropriate fluorescent pH probes to study intracellular pH. (3) Use these two technical developments to investigate the mechanisms of intracellular proton buffering of extrinsic and intrinsic acid loads.

Methods

A microspectrophotometer has been developed which permits the topological monitoring of either absorbance or fluorescence spectrum from single cells. The basis of the instrument is a modified Leitz microscope. The topological spectral scanning is based on the 0° and 1° order defractions of the transmitted or fluorescence slit image of a cell. Using the zero order a standard slit image of the cell is recorded on a SIT vidicon camera and stored on a video image processing system. The grating is them moved and the first order defraction is collected which then provides an optical spectrum of the slit as a function of position along the long axes of the slit. Storing this in the video processor then permits the determination of the spectral characteristics of each region of the cell along the slit. In addition, the stored spectra can be used to spectrally analyze the whole slit by summing all of the spectra together.

Major Findings

Using the intracellular pH probe 1,4 dihydroxyphallonitrile (DHPN) as a fluorescent indicator we have measured the intracellular pH in the isolated perfused rabbit proximal straight tubule, cultured A6 epithelial cells and rabbit blastocysts. The DHPN is apparently chemically modified in the straight tubule of the rabbit and thus, only directional changes in intracelular pH can be measured in this tissue. No chemical modification was detected in the A6 cells or in rabbit blastocysts. Using the topological mode the intracellular cystolic pH within the A6 cells and blastocysts was homogeneous throughout within the resolution of the dye (0.1 pH). Preliminary studies on A6 cells indicated that the intracellular pH was extremely insensitive to extracellular pH (6 to 8) but could be alkalized or acidified with ammonia.

Proposed Course

To examine the mechanisms involved in the reaction of intracellular pH to extrinsic and intrinsic acid loads. This will be performed by varying the external pH with permeable and impermeable buffers. Intrinsic acid sources will be generated from metabolism via glycolysis and ammonia production.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01262-03 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Non-invasive studies of enzyme catalyzed reaction rates by NMR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Robert S. Balaban	Sr. Staff Fellow LKEM, NHLBI
Others:	Howard L. Kantor	Med. Staff Fellow LKEM, NHLBI
	James A. Ferretti	Research Chemists LC, NHLBI
	Valter A. Saks	Chief, Inst. of Cardiology, Moscow, USSR

COOPERATING UNITS *(if any)*

Institute of Cardiology, Moscow, USSR and Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	1.6	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The reaction rate constants of enzyme catalyzed reactions are being determined under steady-state conditions by non-invasive NMR spectroscopic techniques. The 2D NMR technique permits the simultaneous determination of all of the rate constants involved in a reaction sequence, and explicitly displays them in the form of a 2D plot. In addition, the 2D NMR experiment also provides information on the relative size of the substrate pools involved in the reaction, critical information for determining reaction rates in the compartmentalized cell cytosol, but cannot detect exchanges from very small substrate pools. In contrast saturation transfer permits the monitoring of exchange processes between very small substrate pools, i.e. enzyme substrate complexes, and large pools. Four in vitro enzyme systems have been studied; phosphoglucose isomerase, carbonic anhydrase, adenylate kinase and creatine kinase. In all three cases, unique information of both the mechanism and flux characteristics of the reactions have been obtained. Due to the non-invasive nature of these techniques, they were applicable to the determination of enzyme reaction rates within in vivo tissues. We have obtained this rate data from the in vivo brain and leg of anesthetized rats. The creatine kinase catalyzed exchange between CrP and ATP was observed in both tissues, while the ATP hydrolysis-resynthesis rate was too slow to detect in these intact tissues.

Objectives

The object of the in vitro experiments was to determine the enzymatic rate constants and flux behavior of several enzymes which should make important contributions to the steady state exchange of high energy phosphate bonds in vivo. The in vivo 2D NMR experiments were performed to investigate the steady state flux through these enzymes within the intact animal.

Methods

All experiments were performed on ^{31}P at 147 mHz and ^{13}C at 95 mHz using a 8 cm bore Nicolete NMR spectrometer. The 2D NMR technique used was basically that described by Ernst and coworkers for simple chemical exchange reactions. We have modified these procedures to study more complex enzyme catalyzed reactions. Basically, kinetic 2D NMR measurements use the inherent frequency label of atomic nuclei to determine the rate of conversion of one molecular species into another. The four enzymes we have studied are: phosphoglucose isomerase, adenylate kinase, carbonic anhydrase and creatine kinase. Saturation transfer was performed using the "DANTE" pulse sequence method to label individual resonances. All enzymes were studied at equilibrium. The reaction rates and concentrations of the substrates were determined from the ^{31}P and ^{13}C NMR resonance of the interconverting molecular species.

The in vivo experiments were performed on Sprague-Dawley rats (110 to 125 grams). The rats were anesthetized with either pentobarbitol (Somnifer) 50 mg/kg or with Inactin 40 mg/kg by intraperitoneal injection. The rats were then secured in a cradle 8.0 cm in diameter. A saddle coil 1.5 cm in length and 1.25 cm in height was wrapped around either the upper thigh or head of the rat. The cradle was then shielded with an aluminum cylinder and the coil tuned to 147 MHz. The cradle was placed in the magnet and the field was shimmed using the water proton signal. Proton linewidths of less than 80Hz were routinely obtained. The 90° magnetization flip was obtained with pulse widths of 50 to 125 μ sec depending on the preparation. All experiments were performed using a single phase detector, without phase cycling.

Major Findings

A method for detecting enzyme substrate complexes not visible in a normal NMR experiment has been developed using saturation transfer NMR. By moving a selective saturating pulse through a spectrum the small enzyme substrate pools can be detected via the transfer of the saturation of the large free pools of substrate and products. Using this procedure we have been successful in directly determining the enzyme-substrate dissociation rates for several of the creatine kinase intermediates. The interference of these enzyme substrate complexes in the determination of bulk ATP $\rightleftharpoons$ CrP and ATP $\rightleftharpoons$ Pi exchange by saturation transfer may partially explain the observed differences between saturation transfer and 2D NMR results in vivo. Bicarbonate and CO_2 are very difficult to monitor independently, in biological fluids. Using ^{13}C NMR we have demonstrated that both HCO_3 and CO_2 can be observed in red blood cells and the unidirectional rate constants of carbonic anhydrase determined. Human red cell carbonic anhydrase was found to increase the exchange of HCO_3 and CO_2 by six-orders of magnitude at 28°C.

Proposed Course

We intend to use the inherent differences in the 2D and saturation transfer NMR experiments to investigate substrate compartmentation in intact tissues as well as isolated organelles such as mitochondria and microsomes.

Publications

Balaban, R.S., H.L. Kantor and R.S. Balaban: In vivo flux between phosphocreatine and adenosine triphosphate detrmined by 2D phosphorous NMR. J. Biol. Chem. 258: 12787-12789, 1983.

Kantor, H.L., J.A. Ferretti and R.S. Balaban: Kinetics of creating phosphokinase and adenylate kinase: A two-dimensional NMR analysis. Biochim. Biophys. Acta (in press), 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01263-03 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Na-K-ATPase efficiency in tumorigenic cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Robert S. Balaban	Sr. Staff Fellow	LKEM, NHLBI
Others:	John P. Bader	Senior Investigator	LTVP, NCI
	Ronald Lynch	Guest Worker	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Pathophysiology, NCI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In several tissues a coupling between glycolysis and Na+,K+-ATPase has been observed. The purpose of these studies was to investigate the coupling of glycolysis and Na+,K+-ATPase in Rous transformed Hamster cells, Ehrlich ascites tumor cells and A6 cultured cells. The rate of Na+,K+-ATPase was estimated by the initial rate of ouabain-sensitive K+-influx after K+ reintroduction to K+-depleted cells. Experiments were performed on HTcBH and Ehrlich cells producing ATP via oxidative phosphorylation alone (i.e. lactate sole substrate), glycolysis alone (i.e. glucose as substrate in the absence of oxygen or with antimycin A), or glycolysis and oxidative phosphorylation (i.e. glucose as substrate in the presence of oxygen). The cells produced ATP at approximately the same rate under all of these conditions, but the initial rate of K+-influx was approximately two-fold higher when ATP was produced from glycolysis. Changes in cell Na+ due to other transport processes related to glycolysis, such as Na+-H+ exchange, Na+-glucose cotransport and K+-H+ exchange were ruled out as mediators of this effect on Na+,K+-ATPase. These data suggest that glycolysis is more effective than oxidative phosphorylation in providing ATP to Na+,K+-ATPase in these cultured cells.

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Objectives

The objective of this project was to determine in cultured cell lines the efficiency of Na-K-ATPase, the relative amount of energy used by Na-K-ATPase within these cells, and what energy metabolism pathway may be specifically coupled to Na-K-ATPase.

Materials and Methods

Four cell lines are being used in this study, Ehrlich ascites tumor cells, hamster (HtcBH) and chicken embryo cells transformed with Rous sarcoma virus and A6 cells derived from the toad kidney. Oxygen consumption was measured polargraphically. Simultaneously lactic acid production was determined using extracellular yeast LDH and cyt C as optical indicators of extracellular lactate. Intracellular ATP, CrP, inorganic phosphate and pH is determined from ^{31}P NMR spectra of the cells either grown attached, or in suspension, in a perfused hollow fiber apparatus within a Nicolet wide bore 360 NMR spectrometer. Net K^+ fluxes were determined using an extracellular K^+ electrode and net ^+H fluxes were determined using a pH stat device. Intracellular pH was also determined using a fluorescent intracellular pH probe, 1,4 dihydroxyphallonitrile.

Major Findings

The addition of glucose to K^+-depleted HTcBH or Ehrlich ascites cells increased the rate of ouabain sensitive K^+ uptake from reintroduced K^+ and also increased net uptake of K^+ from low K medium equilibrated with the cells. To cause these effects, glucose had to enter the cell and be metabolized through the glycolytic pathway. Alpha methyl glucose did not mimic the glucose effect and IAA blocked it. Several membrane transport processes can be associated with glucose uptake and metabolism such as; Na^+-glucose cotransport, Na^+-H^+ exchange, lactate efflux and K^+-H^+ exchange efflux. All of these processes would increase K^+ influx either directly (i.e. K^+-H^+ exchange) or indirectly via an increase in intracellular Na^+. However, all of these processes were experimentally ruled out as possible mediators of this effect.

Proposed Course

These results suggest that glycolysis is more effective than respiration in delivering ATP to Na-K-ATPase which is not unique to these cell culture lines. Smooth muscle cells and red blood cells previously were found to display a similar behavior. The cells in culture offer a unique advantage since large populations of cells can be grown and isolated plasma membrane vesicles or ghosts made to investigate the role of glycolysis in ATP production for consumption by Na-K-ATPase. Specifically, the possibility of a membrane pool of ATP, ADP and P_i immediately available to Na-K-ATPase and to glycolytic enzymes associated with the plasma membrane will be investigated.

Publications

Balaban, R.S. and J.P. Bader: The efficiency of (Na^+ + K^+)-ATPase in tumorigenic cells. BBA 730:271-275, 1983.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

OFFICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01264-03 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Apical sodium uptake in cultured kidney cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Sarah Sariban-Sohraby	Visiting Fellow	LKEM, NHLBI
Others:	James Turner	Visiting Associate	LKEM, NHLBI
	Maurice B. Burg	Chief	LKEM, NHLBI
	Dale Benos		Harvard Medical School
	Ramon Latorre		Harvard Medical School
	John Johnson	Walter Reed Army Medical Research Center	
	William Wiesmann	Walter Reed Army Medical Research Center	

COOPERATING UNITS (if any)

Harvard Medical School, and Walter Reed Army Medical Research Center

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have developed a method for making functional apical plasma membrane vesicles from cultured toad kidney cells. Sodium uptake into the vesicles has been studied, as well as the mechanism by which aldosterone increases the permeability of the sodium channels. The sodium channels have been reconstituted from the vesicles into planar lipid bilayers and the properties of single channels have been determined.

654

Objective

An important part of the control of renal sodium excretion is modulation of sodium reabsorption in the cortical collecting ducts by aldosterone. One of the actions of aldosterone is to increase the permeability of the apical membranes of these cells. Previously, we identified this action of aldosterone in cultured toad kidney cells (A6). The present experiments were aimed at characterizing the channels by which sodium permeates the apical membranes of A6 and the mechanism by which aldosterone increases their permeability.

Methods

The procedures for growing the A6 cells and for preparing the apical membrane vesicles were described in previous reports, as was the method for reconstituting the channels into planar lipid bilayers and studying the electrical characteristics of a single channel.

Major findings

(1) Sodium uptake into the vesicles was inhibited by low concentrations of amiloride which is characteristic of sodium channels in tight epithelia and therefore confirms the identity of the sodium channels in the vesicles. When aldosterone was added to the cells from which the vesicles were prepared, the sodium uptake doubled.

(2) Aldosterone is known to induce transcription of messenger RNAs that translate to specific, but as yet unidentified, proteins. Some of these proteins are presumably responsible for the increase in sodium channel activity following aldosterone. Methylation of membrane proteins or phospholipids has been suggested as the mediator of the effect. In support of this theory methylation of membrane phospholipids and proteins in the vesicles by addition of S-adenosyl methionine caused the sodium uptake into the vesicles to increase to the same extent as aldosterone. The effect was prevented by inhibitors of methylation and was not additive to the effect of aldosterone.

(3) The single sodium channels reconstituted into planar lipid bilayers were found to have the following properties: Conductance 40 pS, selectivity for sodium much greater than for chloride, selectivity for sodium 2-3 times potassium, prolonged open times with both short and long closed times, and inhibition by characteristically low concentrations of amiloride. There was a dose-dependent decrease in single channel conductance when amiloride was added to the same side that the vesicles were but a decrease only in the open time when amiloride was added to the other side.

Proposed course

Project completed.

Publications

S. Sariban-Sohraby, M. Burg, W. Weismann and J. Johnson. Methylation increases sodium flux into epithelial apical membrane vesicles. Possible involvement in the action of aldosterone. Science (in press).

S. Sariban-Sohragy, R. Latorre, M. Burg, L. Olans and Dale Benos. Amiloride-sensitive epithelial Na^+ channels reconstitued into planar lipid bilayer membranes. Nature 308:80-82, 1984.

S. Sariban-Sohraby, R.J. Turner, and M. Burg. Alsoterone stimulates sodium uptake by apical membrane vesicles from A6 cells. J. Biol. Chem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01265-02 KE

PERIOD COVERED

October 1, 1983 to December 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Epithelial Fluid Transport

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Kenneth R. Spring	Physiologist	LKEM, NHLBI
Others:	Peter Koch Jensen	Guest Worker	LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.50	.50	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epithelial cells swell when the Na-K pump is inhibited with ouabain. The swelling is due to NaCl entry into the cell across the apical membrane. NaCl entry into the cell was shown to be regulated by a feedback system which depended on the concentration of intracellular sodium.

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Objectives

The primary goal of this investigation is elucidation of the mechanism of fluid absorption by epithelia. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use an online, real time system for measuring the size and shape of the cells. Based on changes in cell volume we are able to demonstrate regulation of the rate of entry of NaCl into epithelial cells across the apical membrane.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties and variation in solution composition. The chamber is placed on the stage of an inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations.

Major Findings

The rate of NaCl entry into gallbladder epithelial cells was determined by measurement of the rate of cell swelling following inhibition of the cell Na-K ATPase by application of ouabain. The swelling was caused by NaCl entry into the cells across the apical membrane because it could be prevented by lowering the Na or Cl concentration of the bathing solution to 10 mM. When the cells are exposed to ouabain for long periods of time, the initial swelling period is followed by slow cell shrinkage. We showed that the cessation of cell swelling was due to inhibition of the apical NaCl entry process by a feedback mechanism. In addition the cell shrinkage was shown to be due to KCl loss from the cell across the basolateral cell membrane. This KCl loss was activated by the cell swelling and involves calcium ions.

Proposed Course

Project completed.

Publications

Jensen, P.K., R.S. Fisher and K.R. Spring. Feedback regulation of NaCl entry in Necturus gallbladder. J. Membr. Biol. (submitted).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01266-02 KE

PERIOD COVERED

October 1, 1983 to March 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epithelial Cell Volume Regulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Kenneth R. Spring	Physiologist	LKEM, NHLBI
Others:	J. Kevin Foskett	Staff Fellow	LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.75	.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The control of epithelial cell volume was studied by disturbing the cell cytoskeleton or interfering with calcium activated processes. Volume regulatory decrease was shown to require an intact cytoskeleton as well as calcium dependent processes. Volume regulatory increase was independent of cell calcium and did not require an intact cytoskeleton.

Objectives

The primary goal is to establish the mechanism by which epithelial cells activate volume regulatory transport processes.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties and variation in solution composition. The chamber is placed on the stage of an inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations.

Major Findings

Agents which disturb intracellular microfilaments prevent volume regulatory decrease which follows osmotic cell swelling but do not interfere with the volume regulatory which follows osmotic cell shrinkage. Calcium seems to be involved in volume regulatory decrease because this process was sensitive to a variety of drugs which interfere with calmodulin or calcium activated processes. No calcium dependence was seen for volume regulatory increase.

Proposed Course

We are developing microscopic techniques for measuring intracellular calcium and hydrogen ions utilizing the fluorescence of specific dyes. We will also attempt to measure intracellular Ca^{++} and H^{+} with microelectrodes.

Publications

Foskett, J.K. and K.R. Spring. Calcium and microfilaments in epithelial cell volume regulation. Federation Proceedings 43(3):447, 1984 (abstract).

Foskett, J.K. and K.R. Spring. Involvement of calcium and cytoskeleton in gallbladder cell volume regulation. Am. J. Physiol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01269-02 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Single channel conductance measurements

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Masahiro Yanase	Visiting Fellow	LKEM, NHLBI
Others:	Anne Walter	Staff Fellow	LKEM, NHLBI
	Robert Blumenthal	Research Chemist	LMB, NCI
	Maurice Burg	Investigator	LKEM, NHLBI
	Joseph S. Handler	Investigator	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Mathematical Biology, NCI, NIH

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section, LKEM, NHLBI

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The response of sodium transport to hormones is studied in a cultured epithelium. Transport is assayed by measuring amiloride-sensitive sodium uptake into apical membrane vesicles prepared from A6 cells, a cultured cell line derived from toad kidney. Amiloride sensitive sodium channel will be examined for specific binding of 3H-labeled-Benzamyl. The hormones to be studied are aldosterone and antidiuretic hormone.

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Objectives

The study is intended to elucidate the cellular mechanisms involved in the stimulation of sodium transport by mineralocorticoids and antidiuretic hormone, especially the steps after the ligand binding to receptor. Transcellular sodium transport of A6 cells is modulated by these hormones and the major site of action of these hormones is considered to be at apical sodium entry. Specific objectives are: (1) to develop and characterize an apical membrane preparation suitable for studying post receptor changes in response to the hormones, (2) to determine if increased Na influx is due to increased number of entry sites synthesized de novo, increased activity of cryptic sites or changes in conductivity or selectivity of existing sites, (3) to test hypotheses that Na fluxes are modulated by changes in lipid methylation state or fluidity, specific protein phosphorylation or methylation.

Methods

Apical membrane vesicles are prepared by sucrose density gradient centrifugation of homogenates prepared from cultured A6 cells grown on Millipore filters. Sodium transport is assayed by rapid filtration technique under equilibration exchange conditions using ^{22}Na. ^{3}H-labeled Benzamil binding to isolated apical membrane is measured with ultra centrifugation methods.

Major Findings

(1) Techniques for measuring sodium transport into apical membrane vesicles and monitoring marker enzyme activity are established. (2) Apical membrane vesicles prepared by sucrose density gradient centrifugation give higher specific sodium transport activity than those prepared by Ca_{22} precipitation methods. Both vesicle size and the effect of divalent cations on ^{22}Na flux are factors in this result. (3) Apparent binding constant (Kd) of Benzamil to apical membrane vesicles is 63 nM and maximal binding is 22 pmoles/mg protein. This drug will be used to count sites and to follow sodium channel activity.

Proposed Course

(1) Amiloride-inhibitable sodium uptake will be characterized to establish its characteristics regarding charge conductance and ion selectivity, and to demonstrate that uptake is not merely binding. (2) The isolated apical membrane vesicles will be incubated with ATP and cAMP dependent protein kinase and the changes in sodium transport activity will be monitored. Phosphorylated protein will be analyzed by SDS polyacrylamide gel electrophoresis and radioautography. (3) The isolated apical membrane vesicles will be incubated with s-adenosylmethionine and the changes in sodium transport will be monitored. Transmethylation of phospholipid and protein carboxymethylation will also be studied. (4) The effect of carboxy reacting reagents on sodium transport activity will be studied. (5) We will measure the changes of Benzamil binding induced by aldosterone and antidiuretic hormone treatment.

Publications

None.

PROJECT NUMBE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01270-02 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

14-N NMR spectroscopy of mammalian tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

P.I.:	Robert S. Balaban	Sr. Staff Fellow	LKEM, NHLBI
Others:	Mark Knepper	Med. Staff Fellow	LKEM, NHLBI
	Serena Bagnasco	Visiting Associate	LKEM, NHLBI
	Maurice Burg	Investigator	LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

14N NMR data from the kidney and liver have indicated that significant amounts (50 mM) of trimethylamines are present in these tissues. In the kidney medulla these concentrations reached 100 mM. This high concentration of trimethylamines has previously not been generally known and the role of these cations in the metabolism and osmotic balance of these cells is unknown. We have begun to develop alternate techniques to determine the constituents which make up total trimethylamine found in 14N NMR. The available methods to characterize the different trimethylamines such as betaine, choline, TMA, TMAO, phosphylcholine, glycerol-phosphorylcholine etc. have proven to be very unsatisfactory especially with regard to betaine. We, therefore, are developing a new assay for these compounds based on the very high K+ electrode response to trimethylamines after separating the different trimethylamines on a Dowex ion exchange column.

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Objectives

(1) To develop a reliable assay for determining the various trimethylamines which make up the ^{14}N NMR detected trimethylamine peak. (2) To investigate the effects of the animal hydration state on trimethylamine content.

Methods

All NMR experiments were performed at 26 mHz on a Nicolet NT-360 wide-bore spectrometer. In vivo experiments were carried out on phenobarbital anesthetized rats (50 mg/kg i.p.) secured in an animal cradle 7.5 cm. in diameter specially designed to fit within the magnet. A six turn surface coil of 1.5 cm in diameter made of 1.25 mm insulated copper wire was placed over the appropriate area of the animal and tuned with variable capacitors to 26 mHz. Once the preparation was within the magnet, the Bo magnetic field was shimmed using the proton free induction decay signal. Ammonia was used as the chemical shift reference in all of these experiments. The field was routinely set to the resonance frequency of H_2O in the in vivo experiments. Sampling ^{14}N at 26.009659 resulted in ammonia being 2.2 ppm from this carrier frequency.

The in vitro experiments on standards and tissue extracts were performed with an 8 turn solenoid, 1.8 cm in diameter made with 1.25 mm diameter copper wire. This coil was tuned to 26 mHz with the sample in place.

Trimehtylamine Assay. Two chemical assays for the trimethylamines have been investigated: (1) triiodine precipitation and (2) choline oxidase reactions coupled to either NADH or peroxide detection. The triiodine precipitation method was found to be unsatisfactory for all of the trimethylamines and was abandoned. The choline oxidase method was found to be useful for choline, phosphorylcholine, and glycerol-phosphoryl choline. However, no acceptable assays were found for betaine, carnitine, TMAO or TMA.

The so called "K^+ sensitive resin", Corning #477317, is 4×10^3 times more sensitive to trimethylamine than K^+. Using this property we are currently separating the trimethylamines on an ion exchange column and then detecting the trimethylamines using the K^+ electrode. We have shown that this electrode can detect choline, betaine, TMA, TMAO and acetylcholine at concentrations as low as 1 μM in 2.5 M HCl, 3 M NaCitrate or 3 M NaCl elution solutions.

Major Findings

^{14}N NMR data demonstrated that high concentrations of trimethylamines are present in the kidney and liver. In the kidney 20% of the trimethylamine is glycerol phosphorylcholine. Choline and phosphorylcholine concentrations are so low (1 to 3 mM) as not to make only a minor concentration of the total. The concentration of of all three compounds increased after 24 hour water deprivation in rats. The major constituent of this trimethylamine pool is stull unknown. We speculate that it may be betaine based on the high activity of choline oxidase in kidney tissue. The new assay system being developed for betaine should answer this question.

Proposed Course

To determine the composition of the trimethylamine pool in the kidney and liver. To investigate the role of these compounds in the osmotic regulation in the renal medulla in vivo as well as in cultured medullary cells.

Publications

Nitrogen-14 nuclear magnetic spectroscopy of biological tissues. (1983) R.S. Balaban and M.A. Knepper. Am. J. Physiol. 245:C439-C444.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01272-01 KE

PERIOD

October 1, 1983 to September 30, 1984

Activation of volume regulatory increase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title and laboratory, branch)

P.I.:	Kenneth R. Spring	Physiologist	LKEM, NHLBI
Others:	Donald Marsh	Guest Worker	LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.50	1.50	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Necturus gallbladder epithelial cells activate a quiescent transport system in response to osmotically-induced cell shrinkage. This transport system is located on the apical surface of the cells when the osmotic change is made in the mucosal bath. We show that an osmotic challenge in the serosal bath also elicited transport across the apical membrane indicating that the site of transport is fixed even though the osmolality change occurs on the other surface of the cell.

Objectives

The regulation of cellular volume by Necturus gallbladder epithelial cells has been a subject of interest in this laboratory for several years. Shrinkage of the cells caused by an increase in the osmolality of the mucosal bath has been shown to be followed by spontaneous reswelling to original cell volume. The mechanism of this reswelling (volume regulatory increase) has been studied by several techniques and involves the activation of quiescent transport systems in the apical membrane of the epithelial cell. We had previously observed that an increase of the osmolality of the serosal bath also lead to volume regulatory increase following cell shrinkage. In the present investigation we wished to determine whether the side on which the osmolality was changed had an influence on the location of the transporter responsible for volume regulation.

Methods

Gallbladders were mounted in a chamber designed for the microscopic observation of the epithelial cells. The mucosal and serosal perfusates were supplied from reservoirs connected to the chamber through solenoid activated valves. Rapid changes in the composition of either bath would be achieved by computer controlled switching of the valves. The microscopic images were recorded by a video camera and video disk and analyzed at a later time. The microscope focus was changed by a computer controlled stepping motor. Optical sections of the cells were made and analyzed by quantitative image processing. Cell volume was calculated from the area of each optical section and the focus displacements.

Major Findings

Necturus cells underwent volume regulatory increase after osmotic shrinkage was caused by an increase in the osmolality of the serosal bathing solution. Volume regulatory increase required bicarbonate in the bathing solutions. Removal of NaCl from the mucosal bath blocked volume regulation. Treatment of the apical surface of the epithelium with the inhibitor, DIDS, also blocked volume regulation. These results show that volume regulation proceeds because of the activation of transporters in the apical cell surface even when the osmotic challenge is presented from the opposite side of the cell (the serosal surface).

Proposed Course

The experiments are completed and the results are being prepared for publication.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01273-01 KE

October 1, 1983 to September 30, 1984

Anion exchange in gallbladder epithelial cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	J. Kevin Foskett	Staff Fellow	LKEM, NHLBI
Others:	Kenneth R. Spring	Physiologist	LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.50	.50	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Volume regulation by Necturus gallbladder epithelial cells involves the activation of anion exchange after cell shrinkage. The activity of the anion exchanger was measured by fluorescence microscopy using a fluorescent substrate for the exchanger.

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Objectives

Osmotically induced shrinkage of the epithelium of Necturus gallbladder causes the transient activation of transport systems in the apical cell membrane. Cl enters the cells by anion exchange during the volume regulatory increase which follows osmotic shrinkage. We wished to study the time course of activation of the anion exchanger and to localize the sites of transport within the cells.

Methods

The gallbladder of the amphibian Necturus nuculosus is removed and incubated in a Ringer solution to which 1 mM NBD-taurine, a fluorescent substrate for the anion exchanger, has been added. The cells take up the dye and the tissue is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue. The chamber is placed in the stage of an upright microscope equipped with an epifluorescence illuminator. Laser light is used to excite the NBD-taurine. Fluorescence, transmittance and tissue position are recorded at freuent intervals. After a suitable period the control rate of washout of the fluorescent dye was measured. Then, the apical cell surface was exposed to a hypertonic perfusate and the subsequent rate of dye washout was determined.

Major Findings

The cells accumulate the fluorescent dye in discrete vesicles near the apical surface. There is a slow but steady loss of the dye from the cells under control conditions. The sudden substitution of a hypertonic solution in the mucosal bathing solution causes the cell to shrink and the rate of dye exit to increase transiently. The inhibitor SITS blocks volume regulatory increase and prevents the increase in dye efflux in response to a hypertonic mucosal bath. These results show that volume regulatory increase involves the SITS sensitive activation of anion exchange across the apical membrane of the gallbladder epithelial cells.

Proposed Course

This project is completed. More sophisticated analytical and detection systems will be utilized to obtain both spectral and spatial information on the distribution of fluorescent probes.

Publications

Foskett, J.K. NBD-taurine fluorescence as a probe for anion exchange in gallbladder epithelium. Am. J. Physiol. (submitted).

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

OFFICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01274-01 KE

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hydraulic water permeability of rabbit collecting duct cell membranes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation.)

P.I.:	Kevin B. Strange	Guest Worker	LKEM, NHLBI
Others:	Kenneth R. Spring	Physiologists	LKEM, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.50	1.50	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The loops of Henle and the vasa recta function in the process of water conservation by maintaining high salt and urea concentrations (low water concentration) in the kidney medulla. The collecting duct is the final site in the kidney where either a concentrated or dilute urine can be produced. To retain water the permeability of the collecting duct cell membrane is increased by antidiuretic hormone such that water moves passively from the duct lumen (high water concentration) into the medulla (low water concentration). The measurement of cell membrane water permeability and studies on the control of that permeability and the mechanisms of cell volume regulation in collecting ducts are extremely important for understanding the function of these renal structures.

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Objectives

The research proposed here has two main goals: (a) Studies of epithelial fluid transport have seen renewed interest in recent years with many of the proposed mechanisms being subjects of much controversy. Recently, Persson and Spring were able to measure directly the L_p of mucosal and serosal cell membranes in Necturus gallbladder. The results of these studies demonstrated that the water permeabilities of the cell membranes were large enough such that fluid reabsorption could be explained by simple transcellular osmosis driven by small (1.1-2.4 mOsm) transmembrane osmotic gradients. This investigation eliminated the need for speculation on highly specialized tight junction and lateral space ultrastructure and elaborate models of fluid transport.

Similar studies have not been conducted in other epithelia and are crucial for a better understanding of renal collecting duct function. Measurement of cell membrane Lp will allow us to determine the pathway and investigate the control of fluid reabsorption in different segments of the collecting duct. Is the major pathway for fluid movement paracellular or cellular and does the cytoplasm represent a significant restriction to water flow? Does cell membrane Lp vary in different segments of the collecting duct and with changes in urine osmolality and medullary interstitium hypertonicity? Do high medullary concentrations of urea have any effect on membrane Lp?

Measurements of Lp will also provide us with important information on the function of ADH. Does ADH only alter the permeability of apical membranes or does it also alter the permeability of tight junctions and basolateral cell membranes? Are both the intercalated and principal cells responsive to ADH and do the intercalated cells play any role in transepithelial water movement?

(b) Mechanisms of volume regulation have been studied extensively in single cells over the past 20 - 30 years and more recently in epithelia. In the gallbladder it has been found that the ion transport mechanism involved in volume regulation differ considerably from those mediating transepithelial salt and water movement. Furthermore, these mechanisms are only functional during volume regulation induced by exposure of the cells to anisotonic media.

Cells of the collecting duct are normally exposed to considerable changes in the osmolality of the fluids bathing their mucosal and serosal membranes. Both urinary osmolality and medullary hypertonicity vary dramatically depending on the physiological state of the animal and the degree of diuresis or antidiuresis. Do cells of the collecting duct regulate their volume and is volume regulation influenced by ADH or urea? Does urea function as an intracellular osmolyte as it does in cells of saltwater anurans and elasmobranchs?

Methods

To answer these questions posed above, segments of rabbit cortical and medullary collecting duct will be perfused in vitro. The cortical collecting duct is the easier of the two segments to isolate and perfuse, however, recent studies have also demonstrated the feasibility of perfusing rabbit inner and outer medullary segments. Both cortical and medullary segments have cell outlines which are easily visualized.

Cells of isolated collecting ducts will be studied using a microscope-video system originally developed by Spring and Hope. Briefly, perfused tubules are viewed with an inverted microscope equipped with interference contrast optics. The shallow depth of field of these optics permits "optical sectioning" or viewing of cross-sections of a tubule cell at different distances from serosal to mucosal surface. Cell cross-sections are recorded on video disc using a television camera. The area and perimeter of these sections are determined later from tracings of cell outlines and cell volume is calculated as described by Spring and Hope. The reader should note that high quality video images have recently been made on renal tubules.

Membrane Lp will be calculated as the initial rate of cell volume change following a rapid change in serosal or mucosal fluid composition. Rapid changes in luminal fluid composition will be made using a triple-pipet perfusion system. The Lp is calculated in the absence of a hydraulic pressure difference using the equation.

$$Lp = J_v^o/\sigma\Delta\pi$$

Where J_v^o is the initial rate of fluid flow per unit area of cell membrane, σ is the solute reflection coefficient and $\Delta\pi$ is the transmemrane osmotic pressure difference immediately after solution changes.

Findings

The proposed project will examine the control of apical and basolateral membrane water permeability in cortical and medullary collecting ducts. The equipment for this project requires a modified tubule perfusion apparatus and a computer-driven light microscope and video system. These modifications have been made and the computer programming and equipment interfacing have been completed.

Proposed Course

These experiments are ready to begin. Some problems of temperature control still remain to be solved and then the measurements can be made.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01275-01 KE

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein-induced membrane fusion.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Anne Walter	Staff Fellow	LKEM, NHLBI
Others:	Robert Blumenthal	Research Chemist	LMB, NCI
	Stephen Morris	Expert	NTS, NINCDS
	William J. Vail	Dept. of Biology,	Frostburg State College

COOPERATING UNITS *(if any)*

Laboratory of Mathematical Biology, NCI, Dept. Biology, Frostburg State College, Frostburg, MD., and Neurotoxicology Section, NINCDS

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Fusion events between membranes represent an important catagory of biological dyanamics but the conditions required for controlled fusion events in biological systems have not been defined. We have described fusion of model membranes induced by two basic peptides, apocytochrome c and polylysine, in terms of protein binding, charge requirements and pH dependence. Both proteins promote aggregation and fusion between membranes containing 10% or more negatively charged lipids. The fusion event is pH dependent with maximal rates for a given protein concentration occuring below pH 5.0. Fusion rates are maximal when the charge stoichiometry between lipids and protein is nearly 1:1. Electrostatic binding between two membranes allowing sufficient contact between the bilayers increasing the probability that fusion will occur in response to a trigger such as lowered pH, provides a reasonable model for viral protein triggered fusion events and could apply cellular control processes such as membrane turnover and secretion.

PHS 6040 (Rev. 1/84) GPO 904-917

Objectives

The goals of the project are to describe the conditions necessary for fusion in the presence of charged peptides and to develop a model for the molecular events underlying the process. The requirements for electrostatically induced fusion mediated by a polypeptide will be contrasted with the similar process of calcium-induced fusion in terms of lipid requirements, ligand:lipid charge ratios, pH optima and kinetics. The importance of physical parameters such as vesicle size and charge distribution as well as protein size, flexibility and charge distribution will be described. Finally, the data are being analyzed in terms of a model that predicts the probability of fusion based on charge parameters alone, kinetic models to describe the participating units for aggregation and fusion, and a set of structural models to describe molecular events within the measured energetic parameters.

Methods

Small unilamellar vesicles are prepared by sonication and larger vesicles formed by reverse-phase evaporation of purified lipid mixtures. Small amounts (1 mol% or less) of fluorescently labeled or 3H-labeled phospholipid are incorporated when necessary to follow fusion, aggregation or binding. Aggregation is monitored by centrifugation and OD changes. Leakage of vesicle contents during the fusion event is measured by release of a self-quenching fluorescent dye, 36-Chloride and 14C-inulin efflux. Fusion itself is monitored fluorescently by changes in energy transfer efficiency between two lipid probes as the phospholipids in labeled vesicles are diluted by fusion with unlabeled vesicles. Fusion is also followed by increases in the mean vesicle size and the dispersity of vesicle sizes as seen in freeze fracture electron micrographs. Protein binding is measured using 14C-modified peptides. Protein conformational changes are determined from the circular dicroism spectra in the far uv . The extent of aggregation and fusion are followed by hand mixing experiments in a conventional spectrophotomer and fluorometer respectively. The initial rates of both processes are followed simultaneously in a stopped-flow apparatus as a function of concentration and temperature. Kinetic data are analyzed according to first and second order models. Charge requirements are being described using a probability model assuming normal distributions of binding sites.

Major Findings

In contrast to calcium induced fusion, both peptides fuse vesicles containing small amounts of negatively charged lipids mixed with phosphatidylcholine, a lipid generally considered an inhibitor of fusion. Apocytochrome c, but not polylysine, will fuse vesicles composed exclusively of PC indicating either a strong interaction with the lipid phosphate group or a hydrophobic componant to binding. The extent of fusion increases with increasing relative amounts of protein until a charge ratio between the protein and lipids is about 1:1 suggesting that (1) maximal binding between vesicles and (2) complete charge neutralization are limiting parameters. The size of the peptide is relatively unimportant compared to the charge ratio requirement. Fusion occurs at lower protein concentrations when the pH is decreased to the pKa of the lipids. The increase is greater than predicted by changes in charge ratio alone. Fusion occurs among a limited number of vesicles in the population which is predicted by the probability model assuming a low threshold of net charge on a vesicle

if a protein vesicle complex is a requirement for fusion. Both proteins undergo conformational changes from random coil to at least partially α -helical configurations in the presence of vesicles containing negatively charged lipids. The zwitterionic lipid, PC, has no effect on protein structure. The kinetics of aggregation and fusion can be separated at moderate pH (unlike calcium induced fusion which is aggregation-rate limited) meaning that the energetic parameters of the fusion event itself can be studied.

Significance

An increasing number of cellular processes are found to include fusion events between membranes of intracellular organelles or these organelles and the plasma membrane. Protein insertion, secretion, reabsorbtion and processing of ligand-receptor complexes are all important to cellular dynamics and must occur in a controlled fashion. Calcium promoted fusion may be important but model studies indicate that the lipid requirements are very restricted, that unphysiologically high Ca^{++} concentrations are necessary, and, the event is uncontrolled. Proteins are responsible for viral fusion which appears to be triggered by low pH (e.g. endocytosed viruses fuse when the endosome pH is lowered by the proton pump). The G-protein from vesicular stomatitus virus promotes fusion in model systems at low pH. Electrostatic interactions between proteins and membranes provide a sufficiently general type of binding to be appropriate for fusion, and the observation that the process can be triggered by lowering pH suggest that this type of peptide is a reasonable model for cellular fusagens. At the more theoretical level, no one has been able to describe what happens to the bilayer structure in detail during fusion. We can build reasonable molecular models of the of the proteins interacting with the vesicle surface based on the conformational information from the CD spectra. These pictures coupled with a measurement of the forward rate constant from the stopped flow experiments means there is a chance of testing specfic molecular schemes in terms of the molecular and energetic constraints.

Proposed Course

The model peptide studies described above are being completed. Similar measurements will be made with viral proteins and peptide fragments (VSV G-protein and the active peptide from Sendai virus) and data analyzed in terms of the protein structures.

Publications

Walter, A., D. Margolis, and R. Blumenthal. (1984) Polycation induced fusion of acidic lipid containing vesicles: pH and charge dependence. Biophysical Journal 45:72a.

Mohan, R., A. Walter, and R. Blumenthal. (1984) Interaction of apocytochrome C with lipid vesicles: conformational changes and bilayer fusion are charge dependent. Abstract at 8th International Biophysics Congress, August, Bristol, UK.

Annual Report
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1983 through September 30, 1984

The Laboratory of Molecular Cardiology is investigating the regulation of contractile proteins in muscle and non-muscle cells. These studies include the regulation of contractile proteins in smooth muscle, cardiac and skeletal muscle as well as platelets and other non-muscle cells. Sources of contractile proteins include adult, fetal and embryonic muscles as well as muscle cells grown in tissue culture. The purpose of these investigations is to understand the role of the ubiquitous proteins, actin and myosin, in generating contractile activity in muscle and non-muscle cells, and to determine what factors regulate this contractile activity.

Smooth Muscle and Human Platelet Myosin Light Chain Kinase: (M. Nishikawa). The calcium-activated, phospholipid-dependent kinase, protein kinase C, was found to phosphorylate smooth muscle heavy meromyosin at a different site on the 20,000-dalton light chain of myosin than does the enzyme myosin light chain kinase. Phosphorylation of smooth muscle heavy meromyosin by protein kinase C, following phosphorylation by myosin light chain kinase, results in a decrease in the actin-activated MgATPase activity of heavy meromyosin. The decrease in the actin-activated MgATPase activity appears to be due to a seven-fold increase in the K_m actin for HMM that has been phosphorylated by both kinases compared to HMM that has only been phosphorylated by myosin light chain kinase. The decrease in the actin-activated MgATPase activity correlates well with the extent of the additional phosphorylation of HMM by protein kinase C following initial phosphorylation by myosin light chain kinase.

Myosin Phosphorylation in Intact Smooth Muscles: (P. de Lanerolle). Affinity-purified antibodies to turkey gizzard smooth muscle myosin light chain kinase were used to study the immunological properties of myosin light chain kinase isolated from turkey gizzard, bovine tracheal smooth muscle, and human platelets. Immunoprecipitation experiments demonstrated that avian and canine smooth muscle myosin light chain kinases have molecular weights of 130,000 and 150,000, respectively. When all three myosin light chain kinases were subjected to limited digestion by S. aureus V8 protease a number of different peptides were identified. On the other hand, inhibition of the catalytic activity of all three myosin light chain kinases occurred at approximately the same antibody:kinase ratio. These data demonstrate that the myosin light chain kinases are an immunologically heterogeneous group of proteins, but that the regions required for catalytic activity are conserved in all three enzymes despite changes that have appeared to occur in other regions of the enzymes.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction: (J.R. Sellers). In order to identify possible intracellular binding sites for myosin light chain kinase as well as a number of smooth muscle phosphatases the binding constants of these enzymes to actin and myosin were measured in collaboration with Dr. Mary D. Pato, University of Saskatchewan. Myosin light chain kinase bound well to both actin

(K_B = 2.5 x 10^5 M^{-1}) and myosin (K_B = 1.3 x 10^6 M^{-1}). The presence of tropomyosin increased the binding constant for myosin light chain kinase to actin by a factor of three. The presence of calcium and calmodulin resulted in a three-fold decrease in the binding constant of myosin light chain kinase to both actin and myosin. Myosin light chain kinase did not bind to phosphorylated myosin. Two of the phosphatases investigated bound to myosin and this binding was greatly increased when the myosin was thiophosphorylated (K_B = 1 x 10^7 M^{-1}). These studies indicate that all of the elements required for myosin regulation are capable of binding to the thick filaments while myosin light chain kinase may also be bound to actin filaments.

The enzyme, myosin light chain kinase, has been isolated from the horseshoe crab, Limulus. Limulus myosin light chain kinase is dependent upon calcium and calmodulin for enzymatic activity and is very specific for myosin or the isolated light chains of myosin. The K_m for calmodulin is 6 nm and the V_{max} and K_m for phosphorylation of isolated Limulus light chains are 15.4 μM/min·mg and 15.6 μM, respectively.

A technique was developed for determining the light chain binding site on myosin by incubating labelled light chains with polyacrylamide gels containing myosin, or its subfragments, which have been fixed and extensively washed to remove SDS. This technique shows that both types of myosin light chains bind to a region of the myosin head located about 76,000 to 100,000 daltons in sequence from the amino-terminal end of the molecule.

The ability of myosin-coated beads to move on an actin substratum of the algae Nitella was studied in collaboration with Dr. Michael Sheetz, University of Connecticut Health Center. It was found that the movement of the beads required that the myosin be phosphorylated and this movement could be stopped by dephosphorylation using a purified phosphatase isolated from smooth muscle.

The Intracellular Biochemical Regulation of Myocardial Contractility: (M.A. Movsesian). In order to determine whether a dihydropyridine with positive inotropic properties would have a potentiating (rather than an inhibitory) effect upon calmodulin-dependent enzyme activity in vitro, studies were undertaken with a new drug BAY K 8644. BAY K 8644 inhibited calmodulin-dependent turkey gizzard myosin light chain kinase activity in vitro with an IC_{50} of 80 μM. In contrast, when myosin light chain kinase was rendered calmodulin-independent by limited proteolysis, drug inhibition was markedly diminished. BAY K 8644 inhibition was additive with that of the negative inotropic dihydropyridine. These results indicate that the inotropic effects of dihydropyridine in smooth muscle are unrelated to their calmodulin antagonistic properties.

The dihydropyridine Ca^{2+}-antagonists were found to stimulate Ca^{2+} uptake in canine cardiac sarcoplasmic reticulum in vitro by increasing the ATP efficiency of Ca^{2+} transport. This effect was also found not to derive from the calmodulin antagonistic properties of the drugs.

The Ca^{2+}-activated, phospholipid-dependent protein kinase, protein kinase C, was found to catalyze the phosphorylation of a 27,000-dalton membrane protein, phospholamban, in canine cardiac sarcoplasmic reticulum preparations. This phosphorylation is associated with a two-fold stimulation of calcium uptake by cardiac sarcoplasmic reticulum which is similar to that seen following phosphorylation by an endogenous calmodulin-dependent protein kinase or by the catalytic subunit of cyclic AMP-dependent protein kinase. Two-dimensional peptide maps of the tryptic fragments of phospholamban indicates that the three protein kinases differ in their selectivity for sites of phosphorylation; however, one common peptide appeared to be phosphorylated by all three protein kinases. These findings suggest that protein kinase C may play a role in regulating the intracellular calcium concentration of cardiac cells.

Studies on the Structure and Function of Myosin Light Chain Kinase: (M. Elizabeth Payne). The structure of smooth muscle myosin light chain kinase is being investigated using selective proteolysis. Studies have been undertaken to characterize, as well as determine the amino acid sequence, of the site phosphorylated by cyclic AMP-dependent protein kinase as well as the calmodulin-binding domain of the enzyme. In a separate series of experiments, an effort is being made to generate active subfragments of myosin light chain kinase which no longer require the presence of calcium-calmodulin and are no longer regulated by phosphorylation. New studies are being carried on in collaboration with Dr. Randall Kincaid, Laboratory of Cellular Metabolism, which make use of a novel derivative of calmodulin which is capable of forming covalent, reversible cross-link complexes with calmodulin-binding proteins.

Immunological Studies of the Regulation of Myosin Function: (M. Schneider). Monoclonal antibodies that recognize three distinct non-overlapping domains within the heavy chain of smooth muscle myosin isolated from turkey gizzards have been characterized. The three domains of the myosin molecule recognized by these monoclonal antibodies are: (1) a 50-kilodalton peptide derived by controlled proteolysis of myosin subfragment-1, the globular head of the myosin molecule, previously found to be associated with actin binding; (2) the carboxy-terminus region of the subfragment-2 portion of the myosin molecule; and (3) a tryptic peptide of light meromyosin previously found to be associated with self-association of myosin filaments.

Molecular Genetics of Muscle Protein: (M. Vahey). A cDNA library of rat uterus mRNA has been constructed using avian myeloblastic virus reverse transcriptase. The cDNA was cloned into the eco R1 site of the expression vector, lambda GT 11, a bacteriophage of E. coli. Upon induction with isopropylthio-beta galactoside, hybrid protein expression in the recombinant yielded at least five positive clones for myosin light chain kinase by the criteria of I-125 protein A immunological cross-reactivity with anti-myosin light chain kinase antibody.

The Regulation of Cardiac Myosin: (L.S. Tobacman). Fetal cardiac myosin S-1, a proteolytic fragment of the myosin molecule retaining enzymatic activity, was separated by ion-exchange chromatography into two isozymes differing in light chain content. One of these two isozymes

contained the fetal light chain not present in adult ventricle; the other contained a light chain which is also present in the adult. The ATPase rate and the actin affinity of the two fetal isozymes were compared to each other and to adult cardiac S-1. The different types of S-1 exhibited identical behavior. These results represent the first comparison between human cardiac myosin isozymes. The results are also consistent with work indicating little or no difference in human fetal and adult ventricular myosin heavy chain.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01665-09 MC

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Smooth Muscle and Human Platelet Myosin Light Chain Kinase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Masakatsu Nishikawa, M.D., Ph.D., Visiting Fellow, LMC, NHLBI
James R. Sellers, Ph.D., Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.2	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein kinase C phosphorylates smooth muscle heavy meromyosin at a different site on the 20,000-dalton light chain than does myosin light chain kinase. Phosphorylation of smooth muscle heavy meromyosin by protein kinase C, following phosphorylation by myosin light chain kinase, results in a decrease in the actin-activated MgATPase activity of myosin.

640

GPO 904-917

Project Description:

Objectives: The phosphorylation of smooth muscle heavy meromyosin (HMM) by the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was investigated. HMM that was unphosphorylated and HMM that had been previously phosphorylated by the Ca^{2+}-calmodulin dependent kinase, myosin light chain kinase were used as substrates.

Methods Employed: Enzyme purification using molecular sieve, ion exchange and affinity column chromatography; ATPase assays; two-dimensional peptide mapping; phosphoamino acid identification.

Major Findings: (1) Protein kinase C phosphorylates the 20,000-dalton light chain of smooth muscle myosin at a different site than myosin light chain kinase. (2) Threonine is the amino acid phosphorylated by protein kinase C, in contrast to serine, which is phosphorylated by myosin light chain kinase. (3) Pre-phosphorylation of HMM by protein kinase C decreases the rate of phosphorylation of HMM by myosin light chain kinase due to a nine-fold increase in the K_m for pre-phosphorylated HMM compared to unphosphorylated HMM. (4) The sequential phosphorylation of HMM by myosin light chain kinase and protein kinase C results in a decrease in the actin-activated MgATPase activity of HMM due to a seven-fold increase in the K_m for actin compared to that for HMM phosphorylated by myosin light chain kinase alone.

Significance to Biomedical Research: Phosphorylation of myosin is a major mechanism for regulating the contractile activity of smooth muscles.

Proposed Course: Most, though not all, of these studies were conducted *in vitro*. Future studies will be aimed at determining the significance of these phosphorylations *in vivo*.

Publications:

1. Naka, M., Nishikawa, M., Adelstein, R.S. and Hidaka, H.: Phorbol ester induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chain. Nature 306: 490-492, 1983.

2. Nishikawa, M., Hidaka, H. and Adelstein, R.S.: Phosphorylation of smooth muscle heavy meromyosin by calcium activated phospholipid-dependent protein kinase. J. Biol. Chem. 258: 14069-14072, 1983.

3. Nishikawa, M., de Lanerolle, P., Lincoln, P.M. and Adelstein, R.S.: Phosphorylation of myosin light chain kinase from tracheal smooth muscle and platelets by cyclic nucleotide-dependent protein kinases. J. Biol. Chem., in press.

4. Nishikawa, M., Sellers, J.R., Adelstein, R.S. and Hidaka, H.: Protein kinase C modulates *in vitro* phosphorylation of smooth muscle heavy meromyosin by myosin light chain kinase. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01785-05 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Myosin Phosphorylation in Intact Smooth Muscles

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Primal de Lanerolle, Ph.D., Staff Fellow, LMC, NHLBI
Masakatsu Nishikawa, M.D., Ph.D., Visiting Associate, LMC, NHLBI
Ruth Felsen, LMC, NHLBI
Yvette Preston, Biologist, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	1.1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have used affinity-purified antibodies to turkey gizzard smooth muscle myosin light chain kinase to study the immunological properties of these enzymes. Immunocytochemical experiments on muscle tissues demonstrated that these antibodies only reacted with antigenic determinants found in smooth muscles, suggesting that smooth and striated muscle myosin light chain kinases are immunologically distinct. Immunoprecipitation experiments demonstrated that avian and canine smooth muscle myosin light chain kinases have molecular weights of 130,000 and 150,000, respectively. The following results were obtained from experiments on myosin light chain kinases purified from turkey gizzard and bovine tracheal smooth muscles and human platelets: (1) a precipitin band only when the antibodies are cross-reacted with the turkey gizzard enzyme; (2) the production of peptides with different molecular weights when the three enzymes are digested with the S. aureus V8 protease and differential binding of the antibodies to these peptides; (3) the binding of only about a third of the antibodies to the tracheal enzyme when compared to the binding of antibodies to the gizzard enzyme on an Elisa assay; (4) the inhibition of the catalytic activity of all three kinases at approximately the same antibody:kinase ratio. These data demonstrate that myosin light chain kinases are an immunologically heterogeneous group of proteins. They also suggest that the smooth muscle and platelet enzymes may have originated from a common genetic ancestor and that regions required for catalytic activity are conserved in all three enzymes despite substantial changes that appear to have occurred in other regions of the enzymes.

Objectives: One of the major objectives of this laboratory is to understand the regulation of smooth muscle contraction through the catalytic activity of myosin light chain kinase (MLCK). Therefore, we have been studying MLCKs purified from smooth muscle and non-muscle cells in order to more fully understand the functional characteristics of these enzymes. We have reported on the biochemical and physiochemical properties of myosin light chain kinase purified from turkey gizzard smooth muscles. We have also reported on the regulation of turkey gizzard, bovine tracheal and platelet myosin light chain kinases by cyclic nucleotide-dependent protein kinases. We now report on the immunological properties of myosin light chain kinases purified from turkey gizzard and bovine tracheal smooth muscles and from human platelets.

Methods Employed: Immunofluorescence, immunoprecipitation and immunodiffusion studies using affinity-purified antibodies to MLCK, peptide map analysis of MLCK's and Western Blot analysis of the peptide maps, enzyme-linked immunosorbent assay (Elisa) to quantitate antibody binding to MLCK's and biochemical experiments on the inhibition of MLCK activity by the MLCK antibodies.

Major Findings:

1. Immunocytochemical, immunoprecipitation and antibody inhibition experiments performed on muscle or muscle extracts demonstrated that antibodies to smooth muscle MLCK do not cross-react with MLCK's in skeletal or cardiac muscle. These data demonstrate that smooth muscle and striated muscle MLCK's are immunologically distinct.

2. Immunoprecipitation experiments demonstrated that smooth muscle MLCK's have heterogeneous molecular weights since the molecular weights differ from species to species. However, smooth muscle MLCK's have the same molecular weight within a given species.

3. Immunodiffusion, Western Blot and Elisa assays suggested the presence of fewer antibody binding sites on MLCK purified from tracheal smooth muscle as compared to MLCK purified from turkey gizzard smooth muscle.

4. Points 2 and 3 suggest that the primary structures of turkey gizzard and tracheal smooth muscle and non-muscle (platelet) MLCK's are different. This was supported by peptide mapping experiments that demonstrated differences in the molecular weights of the peptides generated by proteolytic digestion.

5. Nevertheless, an analysis of antibody inhibition curves of MLCK activity suggests that certain antigenic domains required for MLCK activity are conserved in smooth muscle and non-muscle MLCK's.

Significance: Smooth muscles are essential for maintaining homeostasis. However, the molecular mechanisms that regulate smooth muscle contraction have only recently been elucidated. MLCK appears to play

a critical role in regulating smooth muscle contraction. We have been studying the biochemical and physiochemical properties of this enzyme in order to understand its physiological role. This study represents an extension of our previous work and describes the immunological properties of MLCK's.

Proposed Course: Prepare monoclonal antibodies in order to study the active site of MLCK.

Publications:

1. Donner, L., de Lanerolle, P. and Costa, J.: Immunoreactivity of paraffin-embedded normal tissues and mesenchymal tumors for smooth muscle myosin. Am. J. Clin. Path. 80: 667-674, 1983.

2. de Lanerolle, P., Nishikawa, M., Yost, D.A. and Adelstein, R.S.: Cyclic AMP content, myosin light chain kinase phosphorylation and relaxation of tracheal smooth muscle. Science 223: 1415-1417, 1984.

3. Burger, P.C., de Lanerolle, P., Chandler, D.B., Sanfilippo, A.P. and Klintworth, G.K.: Arteriolar differentiation during corneal revascularization. Fed. Proc., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01786-05 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James R. Sellers, Ph.D., Staff Fellow, LMC, NHLBI
E.V. Harvey, Biologist, LMC, NHLBI
W.A. Anderson, Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Mary Pato, University of Saskatchewan
Dr. M. Sheetz, University of Connecticut Health Center

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism of the phosphorylation-dependent myosin-linked regulation of smooth muscle myosin is being investigated. One aspect of this regulation involves understanding how myosin light chain kinase and phosphatases interact with myosin or actin. To do this we have measured the binding constants of the kinase and phosphatases to actin and to myosin in the hope of identifying their possible intracellular locations.

We have also developed a method for determing light chain binding sites on myosin or proteolytic subfragments of myosin by incubating labelled light chains with polyacrylamide gels containing myosin (or its subfragments) which have been fixed and extensively washed to remove sodium-dodecyl-sulfate. This technique shows that both types of light chains bind to a region of the myosin head located about 76,000-100,000 Da in sequence from the N-terminal end.

A third project utilizes a novel motility assay in which one monitors the movement of myosin-coated beads on an actin substratum which is present in Nitella cells which have been microdissected to remove the cell walls.

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Objectives: The objective of this project is to understand how phosphorylation of myosin regulates the actin-activated Mg-ATPase activity of smooth muscle myosin in vitro which should help elucidate the regulation of smooth muscle contraction in vivo. To attack this problem we study how phosphorylation affects the interaction of smooth muscle myosin (or its subfragments) with actin and how myosin interacts with the kinase and phosphatases which alter the level of light chain phosphorylation.

Major Findings:

(1) Binding of Smooth Muscle Myosin Light Chain Kinase and Phosphatases to Actin and Myosin: Phosphorylation of myosin by myosin light chain (MLC) kinase is required for contraction of smooth muscle. The myosin must be dephosphorylated by a phosphatase for relaxation to occur. There is some controversy as to the intracellular localization of MLC kinase and no data concerning the localization of the phosphatase responsible for this regulation have been presented. In order to identify possible intracellular binding sites of these enzymes, the binding constants of MLC kinase and various phosphatases to actin and myosin were measured in collaboration with Dr. Mary D. Pato, University of Saskatchewan. We find that MLC kinase bound well to both actin (K_B = 2.5 x 10^5 M^{-1}) and myosin (K_B = 1.3 x 10^6 M^{-1}). The presence of tropomyosin increased the binding constant for MLC kinase to actin by a factor of three. The presence of calcium and calmodulin resulted in a three-fold decrease of the binding constant of MLC kinase to both actin and to myosin. MLC kinase did not bind to phosphorylated myosin.

Several phosphatases active against smooth muscle myosin have been isolated. We tested the binding of smooth muscle phosphatase (SMP)-III, SMP-IV, and the catalytic subunit of SMP-I to myosin and actin. SMP-III and SMP-IV bound to myosin and this binding was greatly increased when the myosin was thiophosphorylated (K_B = 1 x 10^7 M^{-1}). SMP-I bound weakly to thiophosphorylated myosin. None of the phosphatases bound to actin. SMP II, a phosphatase not active against intact myosin, did not bind to thiophosphorylated myosin.

These studies indicate that all of the elements required for myosin regulation are capable of binding to the thick filaments while MLC kinase may also be bound to the actin filaments.

(2) Isolation and Characterization of Limulus MLC kinase: Myosin from Limulus, the horseshoe crab, was earlier found to be regulated by a calcium-calmodulin dependent phosphorylation of its light chains in a manner very similar to the regulation of vertebrate smooth muscle. We have now purified the kinase responsible for this phosphorylation from Limulus muscle. Limulus MLC kinase is dependent upon calcium and calmodulin for enzymatic activity and is very specific for myosin or the isolated light chains. The K_m for calmodulin is 6 nM and the V_{max} and K_m for phosphorylation of isolated Limulus light chains are 15.4 umol/min·mg and 15.6 uM, respectively.

SDS-polyacrylamide gel electrophoresis reveals two major bands with M_r's of 39,000 and 37,000 Da. Both of these proteins bind calmodulin (as determined by a 125-I-calmodulin overlay gel) and have enzymatic activity (as determined by partial separation on a non-denaturing polyacrylamide gel). Unlike vertebrate MLC kinases, that from Limulus is not a substrate for cAMP-dependent protein kinase.

(3) Determination of a Light Chain Binding Region of Smooth Muscle Myosin Using a Gel Overlay Technique: The myosin molecule is a hexamer composed of two heavy chains (200,000 Da) whose C-terminal portion form an alpha-helical rod which diverges in the N-terminal half to form two more globular "head" regions. Each head is associated with two types of light chains whose molecular weights in smooth muscle are 20,000 and 17,000 Da.

We have developed an overlay technique in which either 32-P-labelled 20,000-Da light chain or 14-C-labelled 17,000-Da light chain can rebind to the heavy chains of myosins in SDS-polyacrylamide gel following fixation and extensive washing of the gel to remove SDS. This binding occurs even in the presence of 20 mg/ml bovine serum albumin. In order to localize the region of myosin responsible for this binding, we have subdivided the myosin using controlled proteolysis. We find that the light chains rebind to portions of the heavy chain which contain the head such as heavy meromyosin and subfragment-one (S-1), but not to myosin rod, light meromyosin or subfragment-two (S-2). We have further localized the binding region using controlled tryptic digestion of S-1. This generates three polypeptides in smooth muscle S-1: a 29,000-Da N-terminal fragment, a 26,000-Da C-terminal fragment and a 50,000-Da fragment connecting these two. We find that the binding sites for both types of light chains are localized within the 26,000-Da portion. The 26,000-Da smooth muscle fragment is homologous to a 20,000-Da tryptic fragment from vertebrate skeletal muscle. We find that light chains also bind to this skeletal muscle fragment. This localization is consistent with that predicted from other types of evidence. The stoichiometry of rebinding is 0.1-0.4 mol/mol. We are currently trying to subdivide the 26,000-Da peptide in order to more precisely determine the portion of myosin involved in light chain binding.

(4) Analysis of the Motility of Smooth Muscle Myosin-Coated Beads In an In Vitro System: In collaboration with Dr. Michael Sheetz (University of Connecticut Health Center) we have studied the ability of myosin-coated beads to move on the actin substratum of the alga, Nitella. It was found that the movement of the beads required that the myosin be phosphorylated and that this movement could be stopped by dephosphorylation using a purified smooth muscle phosphatase. The phosphorylated myosin-coated beads moved with a velocity of about 0.2-0.4 u/s which is consistent with estimates of the rate of unloaded shortening in smooth muscle. This velocity of bead movement can be compared to the rate of movement of skeletal muscle myosin-coated beads which is 3-5 u/g or about 10 times that with smooth muscle myosin. It is interesting that the ratio of the V_{max} of the skeletal muscle acto-S-1 ATPase to that of smooth muscle S-1 is also about 10.

Smooth muscle myosin is thought to be able to produce a greater force per head in vivo than that produced by skeletal muscle myosin. In this regard, it is interesting that if phosphorylated smooth muscle myosin and skeletal muscle myosin are mixed in equal amounts and added to beads, the rate of movement is that of the phosphorylated smooth muscle myosin.

Proposed Course: We intend to actively study the mechanism of the phosphorylation-dependent regulation of smooth muscle myosin using these and other approaches. We are particularly interested in trying to determine which step in the kinetic cycle for the hydrolysis of MgATP is the step which is directly affected by phosphorylation. We are also interested in determining the topological location of the light chains of myosin with respect to the myosin head.

In addition we plan to extend our studies to include myosin from non-muscle sources such as human platelets or from the brush border of intestinal epithelium. These myosins also seem to be regulated by a phosphorylation of the regulatory light chains.

Publications:

1. Sellers, J.R., Chock, P.B. and Adelstein, R.S.: The apparently negatively cooperative phosphorylation of smooth muscle myosin at low ionic strength is related to its filamentous state. J. Biol. Chem. 258: 14181-14188, 1983.

2. Hammer, J.A. III, Sellers, J.R. and Korn, E.D.: Phosphorylation and activation of smooth muscle myosin by Acanthamoeba myosin I heavy chain kinase. J. Biol. Chem. 259: 3224-3229, 1984.

3. Sellers, J.R. and Pato, M.D. The binding of smooth muscle myosin light chain kinase and phosphatases to actin and myosin. J. Biol. Chem., in press.

4. Sellers, J.R. and Harvey, E.V. Purification of Limulus myosin light chain kinase. Biochemistry, in press.

5. Margosian, S.S., Chantler, P.D., Sellers, J.R., Malhotra, A., Stafford, W.F. and Slayter, M.S. Susceptibility of both isolated and bound light chains from various myosins to myopathic hamster protease. J. Biol. Chem., in press.

6. Sellers, J.R. and Harvey, E.V. Localization of a light chain binding site on smooth muscle myosin revealed by light chain overlay of SDS-polyacrylamide electrophoretic gels. J. Biol. Chem., in press.

7. Sellers, J.R. and Adelstein, R.S. The mechanism of regulation of smooth muscle myosin by phosphorylation. Current Topics in Cellular Regulation, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 04201-03 MC

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intracellular Biochemical Regulation of Myocardial Contractility

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Matthew A. Movsesian, M.D., Medical Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI
Masakatsu Nishikawa, M.D., Ph.D., Visiting Associate, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Molecular Cardiology

SECTION:

INSTITUTE AND LOCATION: National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A. Intracellular Biochemical Effects of Dihydropyridine Ca++/Calmodulin Antagonists.

We have been studying the correlations between the inotropic effects of Ca++-antagonist dihydropyridines and their biochemical mechanisms of action. We have characterized the calmodulin-antagonist properties of these drugs by determining drug inhibition of smooth muscle myosin light chain kinase activity in vitro. We found that dihydropyridines with "Ca++-agonist" properties inhibited a calmodulin-dependent enzyme in a manner similar to and additive with that of their Ca++-antagonist analogues, implying that the inotropic properties of these drugs are unrelated to their calmodulin-antagonist properties.

In a separate system, we demonstrated that dihydropyridine Ca++-antagonists stimulate Ca++ uptake in canine cardiac sarcoplasmic reticulum in vitro by increasing the ATP efficiency of Ca++ transport, and demonstrated also that this effect does not derive from the calmodulin-antagonist properties of the drugs.

B. Regulation of Cardiac Sarcoplasmic Reticulum Ca++ Uptake.

We have shown that a Ca++-activated, phospholipid-dependent protein kinase present in cardiac cells is able to phosphorylate a 27,000-dalton protein in cardiac sarcoplasmic reticulum and stimulate Ca++ uptake. This phosphorylation occurs in at least one site that is different from that phosphorylated by either cAMP- or calmodulin-dependent protein kinases.

Major Findings and Biomedical Significance: (A) We have been engaged in examining the biochemical mechanisms of action of Ca^{++}-antagonist drugs. Dihydropyridine Ca++-antagonists are able to block the influx of Ca^{++} across contractile cell membranes with resultant negative inotropic effects. Several lines of evidence have suggested that the negative inotropic effects of Ca^{++}-antagonists may derive additionally from intracellular actions of the drugs. Many intracellular enzymes involved in regulating contractile function are activated by calmodulin, and we have previously shown that the dihydropyridines, felodipine and nitrendipine, are able to bind to calmodulin and block calmodulin-dependent enzyme activation in vitro. These observations raised the possibility that inhibition of calmodulin-dependent enzyme activation contributes to the negative inotropic properties of Ca^{++}-antagonistic dihydropyridines. German workers recently synthesized a new dihydropyridine having positive inotropic effects competitive with the negative inotropic effects of Ca^{++}-antagonist dihydropyridines. We studied the in vitro effect of this new drug, BAY K 8644, on calmodulin-dependent turkey gizzard myosin light chain kinase activity in order to determine whether a dihydropyridine with positive inotropic properties would have a potentiating rather than inhibitory effect upon calmodulin-dependent enzyme activation. BAY K 8644 inhibited calmodulin-dependent turkey gizzard myosin light chain kinase activity in vitro with an IC_{50} of 80 uM. In contrast, when myosin light chain kinase was rendered calmodulin-independent by limited proteolysis, drug inhibition was markedly diminished. BAY K 8644 inhibition was additive with that of the negative inotropic dihydropyridine felodipine. These results indicate that the inotropic effects of dihydropyridines in smooth muscle are unrelated to their calmodulin-antagonistic properties.

ATP-dependent Ca^{++} uptake by cardiac sarcoplasmic reticulum, a process whereby the intracellular Ca^{++} concentration and hence the contractile state of cardiac muscle cells is regulated, can be stimulated in association with phosphorylation of the sarcoplasmic reticulum protein phospholamban by a membrane-associated calmodulin-dependent kinase. Drugs that bind to calmodulin and inhibit the activation of calmodulin-dependent enzymes might therefore be expected to inhibit sarcoplasmic reticulum Ca^{++} uptake. We examined the effects of four Ca^{++}-antagonists that possess the ability to bind to calmodulin as well as the effect of the calmodulin antagonist trifluoperazine on Ca^{++} uptake and Ca^{++} + Mg^{++}/ATPase activity in canine cardiac sarcoplasmic reticulum. In the presence of 20 uM - 30 uM felodipine and 100 uM - 200 uM nitrendipine, Ca^{++} uptake increased from 69 nmoles/mg-min to 107 nmoles/mg-min and 108 nmoles/mg-min, respectively, with half-maximal stimulation occurring at 7.5 uM and 28 uM, respectively. Ca^{++} + Mg^{++}/ATPase activity was unchanged over the same concentration ranges. In contrast, both Ca^{++} uptake and Ca^{++} + Mg^{++}/ATPase activities were inhibited in the presence of 10 uM - 100 uM trifluoperazine (IC_{50} = 25 uM), 10 uM - 100 uM prenylamine (IC_{50} = 35 uM) and 100 uM - 200 uM verapamil (inhibition insufficient for IC_{50} determination). None of the drugs affected membrane permeability to Ca++ as determined by passive 45-Ca^{++} efflux in the presence of EGTA. Drug inhibition of calmodulin-dependent myosin light chain kinase activation was used as a

direct measure of calmodulin-antagonism, and felodipine, nitrendipine, trifluoperazine, prenylamine, and verapamil blocked this activation at IC_{50}'s of 9.8 uM, 55 uM, 6.4 uM, 31 uM, and 93 uM, respectively. None of the drugs studied, however, had any effect upon endogenous phospholamban phosphorylation in our cardiac sarcoplasmic reticulum preparations. These observations indicate that dihydropyridine Ca^{++}-antagonists stimulate cardiac sarcoplasmic reticulum Ca^{++} uptake in vitro either by increasing the efficiency of the transport process or by inhibiting Ca^{++}-dependent Ca^{++} release, and suggest that these effects do not result from interference with calmodulin-mediated processes. The contribution of these effects to the clinical effects of these drugs remains to be determined.

Proposed Course: We feel this aspect of our project is essentially complete and no further studies are anticipated.

Publications:

1. Movsesian, M.A., Swain, A.L. and Adelstein, R.S.: Inhibition of turkey gizzard myosin light chain kinase activity by dihydropyridine calcium antagonists. Biochem. Pharmacol., in press.

2. Movsesian, M.A. and Adelstein, R.S.: Inhibition of turkey gizzard myosin light chain kinase activity by BAY K 8644. Eur. J. Pharmacol., in press.

3. Movsesian, M.A., Ambudkar, I.S., Adelstein, R.S. and Shamoo, A.E.: Stimulation of canine cardiac sarcoplasmic reticulum Ca++ uptake by dihydropyridine Ca++ antagonists. Biochem. Pharmacol., in press.

Major Findings and Biomedical Significance: (B) Stimulation of Ca^{++} uptake by cardiac sarcoplasmic reticulum has been observed in association with the phosphorylation of the 27,000-M_r membrane protein phospholamban by cAMP-dependent protein kinase and by an endogenous calmodulin-dependent protein kinase. During the past several years an 80,000-M_r protein kinase whose activity is dependent upon the presence of Ca^{++} and phospholipid has been identified. This Ca^{++}-activated, phospholipid-dependent protein kinase (protein kinase C) is ubiquitous in mammalian tissue and has been isolated and purified from bovine heart. We found that protein kinase C is able to catalyze the phosphorylation of phospholamban in a canine cardiac sarcoplasmic reticulum preparation. This phosphorylation is associated with a two-fold stimulation of Ca^{++} uptake by cardiac sarcoplasmic reticulum similar to that seen following phosphorylation of phospholamban by an endogenous calmodulin-dependent protein kinase or by the catalytic subunit of cAMP-dependent protein kinase. Two-dimensional peptide maps of the tryptic fragments of phospholamban indicate that the three protein kinases differ in their selectivity for sites of phosphorylation. However, one common peptide appeared to be phosphorylated by all three protein kinases. These findings suggest that protein kinase C may play a role similar to those played by cAMP- and calmodulin-dependent protein kinases in the regulation of Ca^{++} uptake by cardiac sarcoplasmic reticulum, and raise the possibility that the effects

of all three protein kinases are mediated through phosphorylation of a common peptide in phospholamban. Protein kinase C may therefore play a role in regulating the intracellular Ca^{++} concentration of cardiac cells.

Proposed Course: Further study is being directed at determining the additivity of the effects of phosphorylation by the various kinases and the susceptibility of such effects to dephosphorylation by exogenous phosphatases.

Publications:

1. Movsesian, M.A., Nishikawa, M. and Adelstein, R.S.: Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. Stimulation of cardiac sarcoplasmic reticulum calcium uptake. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04202-03 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Structure and Function of Myosin Light Chain Kinase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. Elizabeth Payne, Ph.D., Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI
William Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Marshall Elzinga, Brookhaven National Laboratory
Dr. Randall Kincaid, Laboratory of Cellular Metabolism, NHLBI

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
1.6	1.1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure of smooth muscle myosin light chain kinase is being investigated. Using selective proteolysis, we are studying the sites phosphorylated by the cAMP-dependent protein kinase and the calmodulin binding domain of myosin light chain kinase. We are also interested in the generation of active subfragments of myosin light chain kinase which no longer require the presence of calcium, calmodulin and are no longer regulated by phosphorylation.

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Objectives: Determination of the amino acid sequence at the sites on myosin light chain kinase phosphorylated by the cAMP-dependent protein kinase.

To isolate and characterize the calmodulin binding domain on myosin light chain kinase.

Methods Employed: Myosin light chain kinase was purified by a method developed in this laboratory. Calmodulin was purified from porcine brain using the method of Watterson et al.. Cross-linking of CaM and myosin light chain kinase was carried out by a modification of the method of Mornet et al. or by the method described by Dr. Kincaid (unpublished results).

Major Findings: Smooth muscle myosin light chain kinase is regulated both by calcium, calmodulin and by phosphorylation. Cyclic AMP-dependent protein kinase can catalyze the incorporation of up to 2 mols P/mol myosin light chain kinase which results in a concomitant decrease in activity. In order to gain a more complete understanding of the regulation and mechanism of action of the enzyme, a detailed analysis of the structure of the protein was initiated. Brief digestion of the phosphorylated kinase with TPCK-trypsin at 0°C results in the generation of a 26,000-dalton phosphopeptide, which contains both phosphorylation sites. The phosphopeptide, as well as an active, calcium, calmodulin-independent fragment, can be resolved using ion exchange chromatography. Digestion of the 26,000-dalton fragment by S. aureus protease yields a low molecular weight, single phosphopeptide which can be resolved using high performance liquid chromatography. In collaboration with Dr. Marshall Elzinga (Brookhaven National Laboratory), the amino acid sequence surrounding the sites phosphorylated by cAMP-dependent protein kinase is being determined.

In addition, studies were initiated to cross-link purified myosin light chain kinase and calmodulin using several different cross-linking agents. With both N-ethyl,N'-(3-dimethylaminopropyl)-carbodiimide-hydrochloride and the dimethyl intermediate, cross-linking did occur; however, the enzyme activity was greatly diminished. Using a novel derivative of calmodulin developed by Dr. Randall Kincaid (Laboratory of Cellular Metabolism, NHLBI) which is capable of forming covalent, but reversible crosslinked complexes with calmodulin binding proteins, cross-linked species were produced which retain full enzymatic activity. Dr. Kincaid and I are currently using this technique to study the calmodulin binding domain of myosin kinase.

Significance: These investigations are directed toward obtaining a greater understanding of the relationships between structure and function of myosin light chain kinase. Myosin light chain kinase is an important enzyme in smooth muscle in that it catalyzes the phosphorylation of the 20,000-dalton subunit of myosin. This phosphorylation is essential for the activity of the actin-activated ATPase of myosin. An understanding of the mechanisms involved in the regulation of myosin light chain kinase is thus essential for understanding how muscles function.

Proposed Course: Development of a method using high performance liquid chromatography to map the sites on myosin light chain kinase phosphorylated by several different protein kinases.

Elucidation of the roles of the individual sites on myosin kinase phosphorylated by cAMP-dependent protein kinase as well as the autophosphorylation site.

Isolation of the calmodulin binding domain on myosin light chain kinase using selective proteolysis of the cross-linked complex.

Completion of the amino acid sequence of the phosphorylated sites. Study of the interaction of the various domains on myosin light chain kinase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04204-03 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunological Studies of the Regulation of Myosin Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Michael Schneider, M.D., Medical Staff Fellow, LMC, NHLBI
James R. Sellers, Ph.D., LMC, NHLBI
Maryanne Vahey, Ph.D., LMC, NHLBI
Yvette Preston, Biologist, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.9	1.3	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have produced monoclonal antibodies that recognize three distinct non-overlapping domains within the heavy chain of smooth muscle myosin isolated from turkey gizzard: (1) a 50-kilodalton peptide derived by controlled proteolysis of subfragment-one (the globular head of myosin) previously found to be associated with actin-binding; (2) the carboxy-terminus of subfragment-two; and (3) a tryptic peptide of light meromyosin previously found to be associated with self-association of myosin into filaments.

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Project Description:

Objective: The role of this project was to generate and characterize monoclonal antibodies to different parts of the smooth muscle myosin molecule.

Methods Used: Hybridoma cell lines were derived by polyethylene glycol induced fusion of P3-X63.AG8 myeloma cells with spleen cells from mice immunized with a partially phosphorylated chymotryptic digest of purified adult turkey gizzard myosin. Antibody specificities were determined by solid-phase indirect radioimmune assay and immunoreplica techniques employing turkey gizzard myosin and its purified subunits heavy meromyosin (HMM) having subfragment-1 (S-1), HMM subfragment-2 (S-2), myosin rod, light meromyosin (LMM) and 20- and 17-kDa light chains.

Results: Three different monoclonal antibodies to myosin were characterized. Antibody 633E9 bound to intact myosin, HMM, and S-1, recognizing a 50-kDa peptide of S-1 previously found to be associated with actin binding. Antibody 420D3 recognized an epitope of S-2 cleaved either from HMM or from rod, which also was detected in the S-2 domain of intact HMM but not in the S-2 domain of intact myosin or rod. Assignments, using purified myosin fragments and one-dimensional peptide mapping, of antibody 633E9 to S-2 and 420D3 to the carboxy-terminus of S-2 were substantiated by the topography of individual [antibody·HMM] complexes visualized by electron microscopy after lowangle rotary shadowing with platinum. Antibody 583E3 recognized an epitope of LMM detected in a 50-kDa tryptic peptide of LMM associated with filament formation. Antibody 583E3 bound the 200-kDa myosin heavy chain in homogenates of 19-day embryonic chicken gizzard, intestine, and aorta, but not pectoralis, cardiac ventricle, cerebellum, or liver, whereas antibody 633E9 bound poorly to myosin heavy chains of embryonic smooth muscle. None of the antibodies bound to myosin purified from avian skeletal muscle or mammalian smooth muscle, to avian non-muscle myosin, or to homogenates of adult rat smooth muscle. Thus, we have produced monoclonal antibodies that recognize three distinct, non-overlapping domains of the heavy chain of avian smooth muscle myosin: heavy meromyosin subfragment-1, subfragment-2, and light meromyosin.

Projected Course: Future studies will entail the characterization of monoclonal antibodies raised to different portions of the myosin molecule.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04205-02 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics of Muscle Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Maryanne Vahey, Ph.D., Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Pamela Benfield, Frederick Cancer Research Facility
Dr. Mark Pearson, Frederick Cancer Research Facility

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using avian myeloblastic (AMV) virus reverse transcriptase we have constructed a cDNA library of rat uterus mRNA. The cDNA was cloned into the Eco R-1 site of the expression vector lambda gt 11, a bacteriophage of E. coli. Upon induction with isopropylthio-beta-galactoside (IPTG), hybrid protein expression in the recombinant yielded at least five positive clones for myosin light chain kinase by the criteria of I-125 protein A immunological cross reactivity with anti-myosin light chain kinase antibody.

Progress is being made on synthesizing a cDNA library of adult turkey gizzard mRNA. We are developing mRNA denaturation techniques to make the gizzard mRNA a better substrate for the AMV reverse transcriptase enzyme.

PHS 6040 (Rev. 1/84)

GPO 904-917

Objectives: The goal of this project is to generate cDNA probes, specific for myosin light chain kinase mRNA. Once identified and characterized such probes can be used to: (1) study the structure and processing of myosin light chain kinase mRNA; (2) isolate and study the structure of the myosin light chain kinase gene from a genomic library; and (3) determine the regulation of myosin light chain kinase gene expression in the context of hormone induction, developmental stage and genetic processes.

Methods: cDNA libraries were made from adult rat uterus mRNA using AMV reverse transcriptase enzyme, S-1 digestion and addition of Eco R-1 polylinkers. cDNA was cloned into the Eco R-1 site of the expression vector lambda gt 11.

Induction of expression of the hybrid recombinant protein product exploited IPTG induction of the Lac Z promoter in the vector. Clones were screened, directly, by affinity-purified myosin light chain kinase antibody reacted with I-125 protein A (from Dr. Primal de Lanerolle, Laboratory of Molecular Cardiology, NHLBI).

Amplification was done on E. coli strain Y 1088 to prevent destruction, by bacterial proteases, of the insert protein. Plaque purification and induction are carried out on E. coli strain Y 1090.

Eco R-1 and Kpn/SST digestions were used to determine insert size and condition.

Annealing and treatment with methyl-mercury were done on turkey gizzard mRNA to extend the secondary structure of the molecule.

Results: The lambda gt 11 rat uterus library is 2000 infectious phage units per mililiter. Approximately, 70-80% of recombinants have inserts. The average base pair size of the inserts, as determined by restriction endonuclease digestion, is 300-600.

Screening with anti-myosin light chain kinase resulted in identification of 5-10 potential myosin light chain kinase clones. Plaque purification is now underway to isolate these positives.

Construction of a synthetic oligonucleotide from amino acid sequence data on turkey gizzard myosin light chain kinase (from Dr. M. Elizabeth Payne, Laboratory of Molecular Cardiology, NHLBI) is also underway. This probe will confirm the clones already reacted with antibody as myosin light chain kinase clones.

Projected Course: In the coming months the positive recombinants will be: (1) isolated; (2) purified; and (3) sequenced. Northern blots of smooth muscle RNA's with myosin light chain kinase translated recombinant cDNA from the probes will be used to identify the mRNA for the myosin light chain kinase gene.

A genomic library for rat will be screened with the cDNA's positive for myosin light chain kinase to isolate the entire myosin light chain kinase gene.

Publications:

1. Condeelis, J.S., Vahey, M., Carboni, J., De May, J. and Ogihara, S.: Properties of the 120,000 and 95,000 dalton actin binding proteins from Dictyostelium discoideum and their possible functions in assembling the cytoplasmic matrix. J. Cell Biol.: July 1984, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04206-02 MC

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Regulation of Cardiac Myosin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Larry S. Tobacman, M.D., Medical Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been investigating the presence and functional importance of cardiac myosin isozymes in man. Myosin subfragment-one (S-1) prepared from fetal hearts was separated into two light chain isozymes. These two isozymes exhibited indistinguishable ATPase activities and actin-binding properties. They also demonstrated the same behavior as myosin S-1 prepared from adult human hearts.

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Project Description:

Objectives: This study seeks to elucidate molecular mechanisms regulating the interaction of the cardiac contractile proteins, myosin and actin. These two proteins produce the force leading to muscle contraction and the modulation of their interaction relates directly to the modulation of cardiac contractility.

Major Findings:

(1) Previous work has demonstrated species-specific developmental changes in cardiac myosin isozymes. Alterations in cardiac myosin heavy chain composition modify the rate of ventricular shortening, as reflected in vitro by changes in the actin-activated Mg-2+-ATPase rate of purified cardiac myosin. In man, however, cardiac myosin isozymes differ in light chain composition and apparently not in heavy chain composition. To study the functional significance of this phenomenon in man, we prepared myosin S-1 from human adult and fetal hearts. Myosin S-1 is a soluble, catalytically active portion of myosin which is better suited for enzymatic studies than intact myosin. The fetal cardiac myosin S-1 was separated by ion exchange chromatography into two isozymes differing in light chain content. One of these two isozymes contained the fetal light chain not present in adult ventricle; the other contained the light chain which is also present in the adult. The ATPase rates and actin affinity of the two fetal isozymes were compared to each other and to adult cardiac S-1. The different types of S-1 exhibited identical behavior. These results represent the first comparison between human cardiac myosin isozymes. The results are also consistent with work indicating little or no difference in human fetal and adult ventricular myosin heavy chains.

(2) The role of myosin phosphorylation in striated muscle, particularly in cardiac muscle, is poorly understood. Much less myosin light chain kinase is present in adult cardiac muscle than in either smooth muscle or in skeletal muscle. Since cardiac myosin isozymes change during development, we studied whether cardiac myosin light chain kinase activity exhibited similar developmental variations. The amount of myosin light chain kinase activity was the same in hearts freshly obtained from fetal lamb, newborn lamb, and adult sheep. This suggests that the significance of cardiac myosin light chain phosphorylation is similar at different stages of development.

(3) Many previous studies have been concerned with the complex interactions of skeletal muscle myosin with regulated actin, i.e., actin plus troponin-tropomyosin. We are studying the corresponding interactions of myosin, actin, and troponin-tropomyosin isolated from the heart instead of from skeletal muscle.

Significance: Recent reports indicate that the human cardiac myosin light chain isozyme found in fetal life also occurs in newborns and reappears in some disease states. Our data on the functional properties of this isozyme pertain directly to cardiac patho-

physiology and to normal newborn physiology. The data also relate to the more general problem of subunit interactions in the ubiquitous protein myosin.

Proposed Course:

(1) We plan to determine whether the effect of Ca-2+ on the cardiac thin filament is to enhance the binding of cardiac myosin S-1.

(2) Cardiac actin-myosin interactions will also be studied using fluorescent probes located on the actin.

(3) A new model system for investigating cardiac myosin will be sought by trying to produce cardiac heavy meromyosin.

Publications:

1. Tobacman, L.S. and Adelstein, R.S. Enzymatic comparisons between light chain isozymes of human cardiac myosin subfragment-1. J. Biol. Chem., in press.

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL, HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1983 through September 30, 1984

The overall objective of the research program of the Molecular Disease Branch is the delineation of the molecular and structural properties of the human plasma apolipoproteins, the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanism involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein synthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins and lipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoproteins, the apolipoprotein composition of which is determined by the laws of mass action. The constituent of the lipoprotein particle which is responsible for the regulation of the lipoprotein particle transport and metabolism is the apolipoprotein moiety. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for the individual plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating metabolism and provides a framework for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal man and in patients with dyslipoproteinemia and atherosclerosis.

Prerequisite to our understanding of the physiological and biochemical role of apolipoproteins in lipid and lipoprotein metabolism is a detailed knowledge of the molecular structure and function of the plasma apolipoproteins. Over the last several years we have systematically evaluated the molecular structure and primary amino acid sequence of apolipoprotein (apo) A-I, A-II, apoC-I, apoC-II, apoC-III, and apoH.

Of particular interest have been recent studies on apoC-II, the principal cofactor for the enzyme lipoprotein lipase, the enzyme which catalyzes the hydrolysis of plasma triglycerides. The complete amino acid sequence of apoC-II from normal subjects has been completed. ApoC-II is a single protein of 79 amino acids with no covalently bound carbohydrate. The sequence of apoC-II from patients with type V hyperlipoproteinemia was similar to apoC-II obtained from normal subjects. Structural variants of apoC-II will be anticipated to be associated with decreased ability to activate lipoprotein lipase and the development of hypertriglyceridemia.

Following the completion of the covalent structure of apoC-II, the total solid phase synthesis of apoC-II was completed utilizing the PAM resin. Synthetic apoC-II was purified to homogeneity and had full cofactor activity when compared to native purified appoC-II. This is the first complete synthesis of a human apolipoprotein and will permit definitive analysis of the structure-function requirements for cofactor activity with lipoprotein lipase.

In order to understand the biosynthesis and metabolism of apoC-II, we have cloned the human apoC-II gene. The apoC-II gene was identified in a human liver cDNA library by screening with synthetic oligonucleotides based on the amino acid sequence of apoC-II. The clone which contained the apoC-II was identified, and the complete nucleic acid of apoC-II mRNA and derived amino acid sequence ascertained. The amino acid sequence of apoC-II determined from the nucleic acid sequence was identical to the sequence of apoC-II derived from the protein amino acid sequence. ApoC-II was shown to be biosynthesized as a 101 amino acid preapoC-II with a 22 amino acid prepeptide. PreapoC-II undergoes intracellular co-translational cleavage to the mature 79 amino acid apoC-II. The elucidation of the pathway for biosynthesis and processing of preapoC-II provides new information on the synthesis and secretion of human plasma apolipoproteins. The availability of nucleotide probes for apoC-II will now permit an analysis of the factors which modulate the biosynthesis and secretion of apoC-II in cell culture.

Patients with a deficiency of apoC-II which clinically is manifested as severe hypertriglyceridemia and a type I lipoproteinemia have been identified. In these patients apoC-II is not present in plasma, and injection of apoC-II into apoC-II-deficient patients results in a prompt reduction in plasma triglycerides. The apoC-II gene has recently been evaluated in two kindred of these patients by restriction enzyme analysis. The apoC-II gene was present, and there are no major insertions or deletions in the apoC-II gene. Additional studies are currently underway to elucidate the molecular defect in patients with apoC-II deficiency.

During the last year major advances have been made in our understanding of the biosynthesis and processing of apoA-I and apoA-II, the two major proteins of high density lipoproteins (HDL). An understanding of the factors which modulate the plasma levels and function of apoA-I and apoA-II and therefore HDL is of major importance because of the established inverse relationship between premature coronary artery disease and plasma HDL levels.

Previously we have established that apoA-I is synthesized as a 267 amino acid precursor protein, preproapoA-I. Eighteen amino acids are present in the prepeptide and 6 amino acids in the propeptide. During biosynthesis preproapoA-I undergoes co-translational cleavage to proapoA-I. ProapoA-I is secreted from the cells and undergoes post-translational extracellular cleavage to mature apoA-I. The enzyme which is responsible for the conversion of proapoA-I to mature apoA-II is calcium-dependent and in plasma, lymph, and at least partially associated with plasma lipoproteins.

Recently we have completed the analysis of the gene for apoA-II. ApoA-II, like apoA-I, is synthesized as a preproapolipoprotein, preproapoA-II. The prepeptide of apoA-II is 18 amino acids and the propeptide is 5 amino acids. The prepeptide is cleaved co-translationally during synthesis, and the propeptide is removed by post-translational cleavage. In marked contrast to apoA-I where the propeptide cleavage is following a gln-gln sequence, the cleavage of proapoA-II occurs following two arginine residues. This site of cleavage is typical of the site of cleavage of intracellular propeptides. However, as is the case with proapoA-I, a large fraction of proapoA-II conversion to apoA-II may occur extracellularly in plasma based on in vitro cell culture studies. The importance of the conversion of the proprotein to mature protein to the physiological function of apoA-I and apoA-II is as yet unknown. Whether the cleavage initiates or terminates a function(s) of these apolipoproteins will be of particular interest.

The elucidation of the pathways for biosynthesis and metabolism of apoA-I and apoA-II has focused attention on potential structural defects in these proteins which will be of functional importance in HDL metabolism. Studies have been performed on three apoA-1 variants which are due to single amino acid substitution in the sequence of apoA-I. These include apoA-I_{Milano}, apoA-$I_{Giessen}$, and ApoA-$I_{Marburg}$. In apoA-I_{Milano} a cysteine replaces an arginine at position 183, apoA-$I_{Giessen}$ a proline for an arginine at 145, and apoA-$I_{Marburg}$ a deletion of the lysine residue 107. Kinetic studies have been performed on each of these apoA-I variants to determine if the structural mutation in apoA-I structure is of importance in the catabolism of the protein. ApoA-I_{Milano} as well as apoA-$I_{Giessen}$ have increased catabolism, whereas apoA-$I_{Marburg}$ is catabolized at the same rate as normal apoA-I. The results from these studies eatablish that structural changes in the A-I apolipoprotein may affect catabolism in vivo and that the effect of a specific amino acid substitution in apoA-I must be determined by specific in vitro and in vivo studies to establish if the structural change in the apolipoprotein is of physiological importance.

Of continuing interest during the last year is the detailed analysis of the isoproteins of apoB. The two apoB isoproteins, designated apoB-100 and apoB-48, are synthesized by the liver and intestine, respectively. We have recently definitively established that only apoB-100 is synthesized by the human hepatocytes in vitro. Normal human hepatocytes were incubated with radiolabeled amino acids, and the secreted apolipoprotein analyzed by ultracentrifugation, immunoprecipitation with monoclonal antibodies and NaDodSO$_4$ gel electrophoresis. The only apoB isoprotein secreted by the human hepatocyte was apoB-100. These studies indicate that the apoB-100 and apoB-48 isoproteins can be used as apolipoprotein markers for lipoproteins secreted by the liver and intestine, respectively, during kinetic studies of lipoprotein metabolism.

A detailed study has been continued on the apoB forms present in patients with various dyslipoproteinemias. ApoB-48 was identified in patients with lipoprotein phenotypes I and V at triglyceride levels of > 1500 mg/dl. ApoB-48 was also identified in plasma of patients with type III hyperlipoproteinemia. One subset of patients with the type III phenotype have apoE deficiency. Patients with apoE deficiency have apoB-48 in all density fractions except HDL. The lipoprotein particles containing apoB isoproteins were separated by affinity chromatography utilizing monoclonal antibodies. Separated lipoprotein particles containing apoB-48 and apoB-100 were isolated consistent with the concept that apoB-48 and apoB-100 were secreted from different tissues on separate lipoprotein particles. Analysis of the kinetics of apoB-48 and apoB-100 permits the determination of metabolism of the liver- and intestine-secreted apoB-containing lipoproteins.

The elucidation of specific physiological and biochemical functions of the apolipoproteins continues to be of pivotal importance in our ultimate understanding of the role of apolipoproteins in lipoprotein structure, function, and metabolism. Based on our current information, apolipoproteins have been shown to be of importance in four general facets of lipoprotein metabolism: 1) cofactor for enzymes (apoC-II and apoH for lipoprotein lipase, apoA-I for lecithin cholesterol acryltransferase); 2) ligand on the lipoprotein particle for interaction with high affinity receptor sites (apoB-100 on LDL and apoE on the chylomicron remnant); 3) exchange protein for phospholipids, cholesteryl esters, and triglycerides; and 4) structural component for the lipoprotein particle (apoA-I for HDL and apoB-100 for LDL).

The determination of these functions has required the development of improved techniques for the evaluation and quantitation of the plasma apolipoproteins. Of importance in clinical screening of patients with dyslipoproteinemia has been the development of a two-dimensional gel system for plasma apolipoproteins. The new system, which involves the use of denaturants and a narrow pH gradient, gives much better resolution than previous systems. This technique will permit the analysis of structural variants of apolipoprotein in plasma, since it separates apolipoproteins both by charge and apparent molecular weight. In addition, the apolipoproteins separated by two-dimensional gel electrophoresis can be quantitated using a computerized gel scanner. This will ultimately provide quantitation data on apolipoprotein isoforms and plasma levels of each of the apolipoproteins.

The direct determination of the plasma levels of apolipoproteins has been performed by radial immunodiffusion or radioimmunoassay. To increase the sensitivity as well as the automation of the quantitation of apolipoproteins, we have initiated the development of the ELISA method. This procedure has been completed for the B-100 apolipoprotein and is currently underway for apoA-I. Ultimately all of the quantitation of the plasma apolipoproteins will be performed by this methodology.

Analysis of the apolipoproteins at the gene level will be initiated by the use of restriction nuclease digestion of white cell genome DNA. This procedure will identify major insertions, deletions, and altered restriction sites in the apolipoprotein genes. This latter technique will be particularly useful in evaluating linkages of apolipoproteins, receptors, and other markers in families and populations with atherosclerosis.

An additional approach to the evaluation of apolipoprotein function is the determination of the factors modulating coordinate control of apolipoprotein biosynthesis. In this regard the chromosomal organization of the plasma apolipoproteins, receptors, and enzymes involved in lipoprotein metabolism will be of particular interest. To determine the chromosomal location of the human apolipoproteins, we have employed hybridization analysis of mouse-human hybrids containing specific human chromosomes. These studies have revealed that apoA-I as well as apoC-III are present on chromosome 11, apoA-II on chromosome 1, and apoC-II as well as apoE on chromosome 19. The chromosomal organization of the plasma lipoproteins gives new insights into the genomic organization of the genes for the plasma apolipoproteins and receptors.

During the last few years a significant focus of the Branch has been an analysis of the interaction of plasma lipoproteins and cellular receptors. Previous studies have shown that the apolipoprotein B is important as the ligand for the interaction of LDL with receptors on peripheral cells, and apoE modulates the uptake of chylomicron remnants by the liver. Recent studies in the Branch have focused on the importance of the liver receptors in lipoprotein metabolism both in normal subjects and patients with dyslipoproteinemia and atherosclerosis. Techniques were developed for culturing of normal human hepatocytes obtained during surgery. In addition, human hepatic membranes were prepared for analysis of binding without cellular uptake.

Initial studies revealed that the binding of apoB-100 was different in human fibroblasts than in liver cells. The liver apoB-100 receptor was different in protease sensitivity, calcium sensitivity, and of lower K_D. In addition,

binding of LDL-apoB-100 was almost totally absent in fibroblasts from patients with familial hypercholesterolemia (FH); however, binding to liver membranes and hepatocytes was reduced by only about 50%. The residual LDL-apoB-100 binding observed in liver from FH patients could be regulated. In patients who underwent a therapeutic portacaval anastomosis, the hepatic binding of LDL-apoB-100 increased markedly, and these changes paralleled those observed in the plasma LDL concentration and hepatic cholesterol and cholesteryl ester content.

Recent studies are consistent with a high affinity receptor for apoA-I and apoA-II of HDL in hepatic membranes. A similar receptor for apoA-I/apoA-II HDL is present on peripheral fibroblasts grown in vitro. The hepatic HDL receptor is modulated by the cellular cholesteryl content and is not displaced by LDL, nitrated HDL, or asialofeutin. The identification and characterization of a receptor for HDL in both peripheral cells and hepatocytes is of major importance, since the transport of cholesterol from peripheral cells to the liver for removal from the body is critical to cellular cholesterol homeostasis and the prevention of atherosclerosis.

The hepatic cellular receptors for lipoproteins containing apoB-100, E, and apoA-I have been evaluated in normal subjects, and patients with abetalipoproteinemia, and FH before and after portacaval shunt. After portacaval shunt apoE binding was increased and apoA-I binding decreased in FH patients. Preshunt the binding of apoA-I containing lipoproteins in FH was identical to the binding observed in normal subjects. The combined results from these studies indicate that there are several distinct receptors for specific apolipoproteins on liver cells which are genetically distinct. The elucidation of these receptors will permit a more definitive evaluation of the regulation of hepatic cholesterol metabolism and lipoprotein biosynthesis.

A unique model to study the importance of the apoB-100 HDL receptor is the Watanabe rabbit (WHHL) which develops spontaneous hyperlipidemia and accelerated atherosclerosis. Previous studies have established that the WHHL rabbit is a good model for the HDL receptor-negative form of homozygous FH. In vitro fibroblast and hepatic membrane LDL receptor studies have revealed that there is not a complete loss of the apoB-100 LDL receptor and that the rabbit model is receptor-defective not absent.

The apoB-100 LDL receptor has been implicated as being important in steroidogenesis tissue. Evaluation of adrenal status in the WHHL rabbit was performed including fasting baseline cortisol and following ACTH stimulation. Fasting baseline levels of adrenal steroids were normal; however, they were decreased when determined after ACTH stimulation. These biochemical studies were paralleled by morphologic differences in adrenal tissue when analyzed by light and electron microscopy. The results of these studies indicate that the WHHL rabbit may have impaired steroidogenic reserve. The extrapolation of these results to patients with homozygous FH suggests that these patients may have reduced steroidogenic reserve during periods of stress.

The intracellular transport, hydrolysis, and biosynthesis of cholesterol continues to be an active area of research within the Branch. The interaction and uptake of LDL by the B-100 receptor initiates a series of biochemical processes leading to the hydrolysis of the cholesteryl esters by acid ester hydrolase with the production of free cholesterol. Free cholesterol downregulates HMG-CoA reductase, the rate-limiting enzyme. in cholesterol biosynthesis and

activates acyl-cholesterol acyltransferase, the enzyme which reesterifies cholesterol to cholesterol ester. Neutral ester hydrolase is the cytosolic enzyme which is responsible for the hydrolysis of cholesteryl esters formed by the action of acyl-cholesterol acyltransferase. New sensitive enzymic techniques have been developed in the Branch for the quantitation of the enzymic activity of acid ester hydrolase and neutral ester hydrolase. These methods were used in the analysis of these enzymes in fibroblasts grown in culture from patients with Wolman's disease and cholesteryl ester storage disease (CESD). Wolman's disease is characterized by steatorrhea, hepatosplenomegaly, absence of acid ester hydrolase, and death in early infancy. CESD is a milder disease with hepatosplenomegaly and reduced levels of acid ester hydrolase. Previous analysis has established that the acid ester hydrolase activity was virtually absent in extracts from fibroblasts from both Wolman patients and CESD. Neutral cholesteryl ester activity was present, not reduced, indicating that the two enzymes are independently synthesized. Additional studies revealed that in CESD the neutral cholesteryl ester activity was increased and may contribute to the milder clinical course observed in CESD patients. Co-culture and cell fusion experiments utilizing fibroblasts obtained from Wolman and CESD patients revealed no cross-correction of enzymic activity. Therefore, unlike several other liposomal storage diseases, the clinical spectrum observed in acid ester hydrolase deficiency cannot be ascribed to a series of defects in enzyme metabolism which can be detected by co-culture or cell fusion techniques.

Recent studies on the plasma lipoproteins in patients with a deficiency of acid ester hydrolase revealed a type II phenotype and markedly reduced HDL levels. These results are interpreted as indicating that lysosomal ester hydrolase activity is important in normal lipoprotein metabolism.

New diagnostic and therapeutic approaches to patients with Wolman's disease have been pursued. The diagnosis of Wolman's disease as well as CESD may be ascertained by the analysis of acid ester hydrolase activity in renal tubular epithelial cells shed in the urine of these patients. This new technique will provide a rapid noninvasive technique to establish the diagnosis of acid hydrolase deficiency. Repletion of Wolman's and CESD fibroblasts with the enzyme acid hydrolase was successful, suggesting that the enzyme is delivered to lysosomes by the mannose-6-phosphate receptor pathway. Thus, enzyme replacement therapy or bone marrow transplantation would ameliorate the lipid accumulation in CESD and Wolman's disease. Recently in collaboration with the Department of Pediatrics at the University of Minnesota, we performed a bone marrow transplant on a seriously ill patient with Wolman's disease. Unfortunately, the child died prior to full engraftment. However, bone marrow transplantation may ultimately prove to be an effective therapy for patients with Wolman's disease.

A second major intracellular enzyme involved in cholesterol metabolism is 3-hydroxy-3-methylglutaryl coenzyme reductase (HMG-CoA reductase). The regulation of the enzymic activity of HMG-CoA reductase has been extensively studied in our laboratory over the last several years. HMG-CoA reductase has been purified to homogeneity from chick, rat, and human liver. In all species now studied, HMG-CoA reductase was shown to be present in enzymically active and inactive forms. The reversible inactivation of HMG-CoA reductase was shown to be due to covalent modification of the enzyme by a reversible phosphorylation-dephosphorylation reaction sequence. The enzyme reductase kinase, which catalyzes the phosphorylation of HMG-CoA reductase, has also been purified to homogeneity. Reductase kinase, like HMG-CoA reductase, was shown to undergo reversible activation-inactivation due to

reversible phosphorylation. The kinase responsible for the reversible phosphorylation of reductase kinase has been designated reductase kinase kinase. Regulation of the enzymic activity of HMG-CoA reductase by a bicyclic cascade system provides a rapid short-term mechanism for the regulation of cholesterol biosynthesis.

Modulation of the degree of phosphorylation and enzymic activity of HMG-CoA reductase has been of particular interest, since this mechanism permits the rapid short-term regulation of cholesterol biosynthesis. The polypeptide hormone, glucagon, and cholesterol have been shown to modulate HMG-CoA reductase activity by changes in the extent of phosphorylation of HMG-CoA reductase. Recently we have completed studies on the regulation of HMG-CoA reductase by mevalonolactone, the product of HMG-CoA hydrolysis. Mevalonolactone was shown to inhibit the activity of HMG-CoA reductase by two general mechanisms. The first involves modulation of the degree of phosphorylation of HMG-CoA reductase catalyzed by reductase kinase. The activity of reductase kinase kinase was increased following mevalonolactone administration (< 20 min). The increase in activity of reductase kinase kinase resulted in an increase in phosphorylation and activation of reductase kinase. The increased activity of reductase kinase catalyzed an increase in the phosphorylation and reduction in activity of HMG-CoA reductase. The overall effect of this activation of the bicyclic cascade system is a reduction in activity of HMG-CoA reductase and decreased cholesterol biosynthesis. The second mechanism involved the reduction in activity of the phosphatase enzymes which are responsible for the dephosphorylation of both reductase kinase and HMG-CoA reductase. The net effect of reduced phosphatase activity is a relative increase in the phosphorylated form of reductase kinase and HMG-CoA reductase, thereby reducing cholesterol biosynthesis. The short-term effect of mevalonolactone is therefore to reduce the biosynthesis of cholesterol by increasing the percentage of HMG-CoA reductase in the inactive or phosphorylated form.

During the last year a new short-term mechanism for the modulation of the activity of HMG-CoA reductase has been elucidated. A second kinase, protein kinase C, which requires calcium and phospholipid, has been shown to reversibly phosphorylate and modulate the enzymic activity of HMG-CoA reductase. Detailed analysis has established that the reversible inactivation of HMG-CoA reductase catalyzed by protein kinase C is due to phosphorylation. Approximately 1 mol of phosphate was incorporated per molecule of enzyme. Dephosphorylation of phosphorylated HMG-CoA reductase was associated with the loss of protein-bound radioactivity and reactivation of enzyme activity. The tumor-promoting phorbol ester, phobol 12 myristate 13 acetate (PMA), stimulated the protein kincase C-catalyzed phosphorylation of HMG-CoA reductase. The increased phosphorylation of HMG-CoA reductase by PMA suggests a possible in vivo protein kincase C-mediated mechanism for the short-term regulation of the activity of HMG-CoA reductase.

The identification of the new protein kinase C system in addition to the reductase kinase-reductase kinase kinase bicyclic cascade system for the modulation of the enzyme activity of HMG-CoA reductase provides new insights into the molecular mechanisms involved in the intracellular regulation of cholesterol biosynthesis.

The synthesis, transport, and catabolism of plasma lipoproteins in normal subjects and patients with dyslipoproteinemia continue to be an active area of investigation within the Branch. One of the informative areas of research over the last several years has been the analysis of the metabolism of apoE. ApoE is coded for by three major alleles, E^2, E^3, and E^4, and several lines of in vitro

and in vivo metabolic evidence suggest that the normal allele is E^3. Previous studies from our Branch have established that the product of the E^2 allele, apoE2, is catabolized more slowly than apoE3. These results are consistent with the delayed catabolism of remnants of triglyceride-rich lipoproteins characteristic of patients with type III hyperlipoproteinemia. The metabolism of apoE2 was also extended to normolipidemic subjects homozygous for apoE2. Initial studies established that normolipidemic apoE2 homozygotes have a two- to threefold elevation of plasma apoE and an increase in cholesterol-rich VLDL. Analysis of apoE metabolism in these subjects revealed that the increase in plasma apoE was due to increase in synthesis.

The metabolism of plasma lipoproteins in normolipidemic apoE2 homozygotes has now been extended to analysis of LDL metabolism. Subjects with apoE2 homozygosity have reduced levels of plasma LDL. Kinetic analysis of radiolabeled LDL isolated from apoE2 subjects in normal and apoE2 normolipidemic individuals revealed that there was an up regulation of the LDL catabolic pathway(s) and a reduced catabolic rate of LDL isolated from the apoE2 subjects. These combined results have indicated that the lipoproteins in normolipidemic apoE2 homozygotes is due to several changes in apolipoprotein-lipoprotein metabolism including increased production of apoE, defective catabolism of lipoprotein remnants, and increased catabolism of an abnormal LDL. These results provide new insights into the factors which are involved in expression of the dyslipoproteinemia associated with the apoE2 phenotype. The development of hyperlipidemia and a type III phenotype in patients with homozygous apoE2 is due to a complex pattern of both primary and secondary defects in lipoprotein metabolism which are significantly affected by environmental, hormonal, and genetic factors.

An important area of research in the Branch continues to be focused on the metabolism of HDL, since HDL has been clearly identified as a negative risk factor for the development of cardiovascular disease. Detailed studies have been carried out on HDL, apoA-I, and apoA-II. Of major importance are our recent studies which have established that proapoA-I is secreted into plasma and lymph and undergoes extracellular post-translational cleavage to mature apoA-I in plasma. Recent data have established that conversion of proapoA-I to mature apoA-I is quantitative, and there is no direct catabolism of proapoA-I. In plasma from fasting subjects, approximately 5% of total apoA-I is proapoA-I.

Of particular interest over the last several years has been the study of dyslipoproteinemic patients with a structural apoA-I variant. Tangier disease, which is characterized by a severe deficiency of HDL and a marked increase in plasma proapoA-I, has been extensively analyzed in our Branch. The marked increase in proapoA-$I_{Tangier}$ in patients with Tangier disease led to the speculation that the defect in Tangier disease was a defect in conversion of proapoA-I to mature apoA-$I_{Tangier}$. Detailed kinetic analysis of radiolabeled proapoA-$I_{Tangier}$ and mature apoA-$I_{Tangier}$ revealed that there was no defect in conversion of proapoA-I to mature apoA-$I_{Tangier}$ but that there was a marked increase in the rate of catabolism of proapoA-I with a normal rate of conversion to mature apoA-$I_{Tangier}$. As a result, the majority of apoA-I in Tangier patients is rapidly catabolized and only a small portion is corverted to mature apoA-$I_{Tangier}$. This change in catabolism of proapoA-I with normal conversion to mature apoA-$I_{Tangier}$ results in the relative increase in the proportion of proapoA-$I_{Tangier}$ in patients with Tangier disease. The structural change in apoA-I in Tangier disease which results in rapid catabolism is currently being investigated as outlined previously.

The study of the metabolism of apoB-48 and apoB-100, the apoB isoproteins secreted by the intestine and liver, respectively, has continued during the last year. These studies have been extended to subjects with homozygous familial hypercholesterolemia (FH) which are characterized by normal triglycerides and VLDL and markedly elevated LDL levels. Kinetic analysis of radiolabeled apoB-48 and apoB-100 containing lipoproteins revealed that the metabolism of apoB-48 was normal and there is no defect in chylomicron remnant metabolism. An analysis of the conversion of VLDL-apoB-48 containing lipoproteins to LDL in FH patients revealed a marked increase in the conversion of VLDL to LDL when compared to normal subjects. These results were interpreted as indicating that the apoB/E or LDL receptor is important in the uptake of VLDL remnants. The loss of this receptor in FH patients results in increased conversion of VLDL to LDL, thereby increasing LDL synthesis. These results provide new insights into role of the apoB/E receptor in lipoprotein metabolism and establish its role in modulating the catabolism of remnants of triglyceride-rich lipoprotein particles.

One of the major aims of the staff of the Branch is the effective treatment of hyperlipidemia with the ultimate goal of reducing blood lipid levels at an early stage of atherosclerosis and preventing premature cardiovascular disease. To this end we have initiated an ongoing outpatient clinical trial for the treatment of patients with hypercholesterolemia and the type II phenotype with neomycin and neomycin-nicotinic combination. Neomycin reduced total and LDL cholesterol levels an average of 20 to 24%, respectively. No ototoxicity, nephrotoxicity, or serious side effects have been detected over a two-year period. Neomycin in combination with nicotinic acid normalized the plasma lipoprotein levels in 92% of the patients with type II hyperlipoproteinemia. This latter regimen provides an effective therapeutic approach to the treatment of patients with type II hyperlipoproteinemia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02010-13 MDB

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Function of Plasma Lipoproteins and Apolipoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.3	2.3	3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human plasma apolipoprotein A-I has been shown to be polymorphic in plasma and lymph. This polymorphism is due to the presence of proapoA-I and mature ApoA-I as well as amide heterogeneity of mature ApoA-I.

Several different structural variants of apoA-I have now been identified. The most important variant is apoA-I-Tangier, which has been isolated to homogeneity. ProapoA-I-Tangier, which is increased in patients with Tangier disease, was shown to have a propeptide sequence which was identical to normal apoA-I. The post-transitional cleavage of proapoA-I-Trangier to mature apoA-I-Tangier was also shown to be normal. The catabolism of apoA-I-Tangier has been previously shown by our laboratory to be markedly increased and the structural change in apoA-I-Tangier which leads to the rapid catabolism is currently being investigated.

The complete covalent structure of apoC-II from normal subjects has been determined. The sequence of apoC-II from normal subjects differs from that of apoC-II from some patients with types IV and V hyperlipoproteinemia. The complete solid phase synthesis of apoC-II has also been completed. Synthetic apoC-II has been purified to homogeneity and activates lipoprotein lipase equivalent to native apoC-II.

Studies have continued on the isolation and characterization of apoB-100 and apoB-48, the apoB isoproteins secreted by the liver and intestine, respectively. Detailed analysis of the apoB-containing lipoproteins in a patient with apoE deficiency has shown that apoB-100 and apoB-48 are in separate lipoprotein particles. ApoB-48 and apoB-100 can therefore be used as apolipoprotein markers for remnants of intestine and liver lipoproteins, respectively.

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Project Description:

Objective:

1) Characterization of apoA-I is found in plasma and thoracic duct lymph.

Methods Employed:

The isoproteins of apoA-I have been isolated from plasma and thoracic duct lymph collected from patients undergoing thoracic duct drainage prior to renal transplantation. Samples are collected in 3 mM EDTA and the chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) separated by preparative ultracentrifugation. Lipoprotein fractions are delipidated with chloroform:methanol (2:1, v/v) and the isoproteins isolated by gel permeation chromatography, $NaDodSO_4$ gel electrophoresis, and/or preparative isoelectrofocusing. The isolated isoproteins are evaluated for purity by analytical $NaDodSO_4$ gel electrophoresis, isoelectrofocusing, and amino-terminal analysis.

Major Findings:

Our studies have established that the two major isoproteins in plasma and lymph, designated apoA-I-1 and apoA-I-3, are proapoA-I and mature apoA-I, respectively. These results were obtained by automated Edman sequence analysis of the isolated apoA-I-1 and apoA-I-3 isoforms. ProapoA-I contains 6 additional amino acids, arg-his-phe-trp-gln-gln, attached to the amino terminus of mature apoA-I. The amino terminal sequence of the two major plasma isoforms apoA-I-3 and apoA-I-4 are identical and establish that they are the mature, 243 amino acid, apoA-I isoprotein. There is a slow conversion of apoA-I-3 to apoA-I-4 in plasma during storage and in plasma during kinetic analysis of apoA-I-3 and apoA-I-4, indicating that the most likely difference between apoA-I-3 and apoA-I-4 is amide heterogeneity; however, other prosthetic group heterogeneity must also be evaluated. Previous studies, outlined in last year's annual report, demonstrated that proapoA-I increased in lymph following fat feeding, suggesting that the intestinal secretion of proapoA-I is modulated by dietary intake.

Objective:

2) Isolation and characterization of variants of apoA-I including apoA-$I_{Tangier}$, apoA-$I_{Giessen}$, and apoA-$I_{Marburg}$.

Methods Employed:

ApoA-$I_{Tangier}$ has been isolated by gel permeation chromatography followed by preparative isoelectrofocusing. Both proapoA-$I_{Tangier}$ and mature apoA-$I_{Tangier}$ were isolated by this procedure. In addition, during the last year an affinity chromatographic procedure utilizing a monospecific antibody against normal apoA-I has been developed.

ApoA-$I_{Giessen}$ and apoA-$I_{Marburg}$ were obtained from Dr. Gert Utermann, Marburg, West Germany, and reisolated by preparative isoelectrofocusing. All isolated

apoA-I variants were analyzed for purity by NaDodSO$_4$ gel electrophoresis and analytical isoelectrofocusing.

Major Findings:

ProapoA-I$_{Tangier}$ and mature ApoA-I$_{Tangier}$ have been isolated to homogeneity by both gel permeation and affinity chromatography followed by preparative isoelectrofocusing. Kinetic analysis in normal subjects and patients with Tangier disease has revealed an increased catabolism of apoA-I$_{Tangier}$, indicating that there is a structural abnormality of purified apoA-I$_{Tangier}$ which leads to rapid catabolism. Detailed characterization of apoA-I$_{Tangier}$ is being completed including structural analysis of the apoA-I$_{Tangier}$ gene and analysis of potential post-translational modification of apoA-I$_{Tangier}$ to establish the molecular defect in Tangier disease.

ApoA-I$_{Giessen}$ has been isolated to homogeneity. ApoA-I$_{Giessen}$ differs from normal apoA-I by a single amino acid substitution of an arginine for a proline at position 143 in the amino acid sequence. As a result of this amino acid substitution, apoA-I$_{Giessen}$ has an additional net + charge. Kinetic analysis of apoA-I$_{Giessen}$ in normal subjects revealed a faster rate of catabolism for apoA-I$_{Giessen}$ than normal apoA-I.

ApoA-I$_{Marburg}$ contains 242 amino acids and differs from normal apoA-I by the deletion of the lysine residue at position 107. ApoA-I$_{Marburg}$ has a net -1 charge difference from normal apoA-I and can be readily identified on isoelectrofocusing. Kinetic analysis of apoA-I$_{Marburg}$ in normal subjects revealed normal catabolism, thereby indicating that the structural change in apoA-I$_{Marburg}$ did not significantly affect its metabolism.

Objective:

3) Amino acid sequence of apoC-II.

Methods Employed:

ApoC-II was isolated from normal subjects and a patient with type V hyperlipoproteinemia. ApoC-II was purifed from delipidated VLDL by gel permeation and ion exchange chromatography, followed by high pressure liquid chromatography. The isolated apolipoprotein was characterized by polyacrylamide gel electrophoresis (pH 9.0), NaDodSO$_4$ gel electrophoresis, isoelectrofocusing, amino acid analysis, and amino-terminal Edman analysis.

The sequence of apoC-II was determined by Edman degradation of intact apoC-II and peptides separated by cyanogen bromide and tryptic cleavage. The Edman degradations were performed on a Beckman 890C sequencer, and the phenylthiohydantoin amino acids determined by high pressure liquid chromatography and mass spectroscopy.

Major Findings:

During the last several years we have investigated the covalent structure of apoC-II isolated from normal subjects. Initial studies established that apoC-II

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was 79 amino acids in length, 1 amino acid longer than the previous structure reported for apoC-II sequenced from patients with type IV and V hyperlipoproteinemia. The covalent structure of normal apoC-II differed in several amino acids including glutamine at positions 2 and 17, serine position 21, tryptophan position 26, and an additional serine at position 21 making the protein 79 instead of 78 amino acids in length.

The sequence of apoC-II was also determined from a patient with type V hyperlipoproteinemia. The sequence was identical to normal apoC-II. The sequence of apoC-II was also determined during the last year by nucleic acid sequence analysis of cloned apoC-II; the derived amino acid sequence was identical to normal apoC-II determined by protein sequence analysis. The combined results from these studies have established the structure of apoC-II from normal subjects. The reason for the heterogeneity in the sequence of apoC-II is not clear. It is of major importance, however, since structural mutations in the sequence of apoC-II may result in C-II apolipoproteins with reduced ability to activate lipoprotein lipase. Clinically, defects in lipoprotein lipase activation will result in hypertriglyceridemia.

Objective:

4) Total solid phase synthesis of apoC-II.

Methods Employed:

The total solid phase synthesis of apoC-II was performed utilizing a Beckman synthesizer model 990. The PAM resin was utilized in the synthesis since this resin has been effectively utilized in the synthesis of large proteins. The following groups were employed in the synthesis: Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Lys (Cl-2), Arg (TOS), His (TOS), and Trp (CHO).

The protein was removed from the resin by HF cleavage, and the formyl group removed from the tryptophanyl residue by 1 M piperidine in 8 M urea.

The synthetic apoC-II was purified to homogeneity by gel filtration, ion exchange chromatography, and high pressure liquid chromatography. The purity of synthetic apoC-II was determined by polyacrylamide gel electrophoresis and Edman sequence analysis.

The ability of synthetic apoC-II to activate lipoprotein lipase was determined utilizing heparin-purified human lipoprotein lipase and a radiolabeled triolein substrate.

Major Findings:

The complete covalent structure of human apoC-II has been synthesized. The synthetic apoC-II was a single band on polyacrylamide gel electrophoresis and a single peak when analyzed by high pressure liquid chromatography. The amino acid sequence of synthetic apoC-II was determined and established that the synthesis was correct.

The biological activity of synthetic apoC-II was evaluated by its ability to stimulate the enzymic activity of human lipoprotein lipase. Synthetic apoC-II had full cofactor activity when compared to native purified apoC-II. The combined results from these studies indicated that the complete structure of apoC-II with full biological activity had been synthesized. This is the first complete synthesis of a human apolipoprotein.

Objective:

5) Characterization of apoB-100 and apoB-48 in normal subjects and patients with dyslipoproteinemia.

Methods Employed:

The two major isoproteins of apoB, apoB-100 and apoB-48, in plasma lipoproteins are separated by 3.5% polyacrylamide gel electrophoresis. The separation of lipoproteins containing apoB-100 or apoB-48 was performed utilizing monoclonal antibodies with specificity against apoB-100 and apoB-48 and against only apoB-100. The isolated lipoproteins were characterized for apolipoprotein and lipid composition by procedures outlined above and in previous annual reports. The lipoproteins containing apoB-100 and apoB-48 were analyzed in patients with type III hyperlipoproteinemia and apoE deficiency.

Major Findings:

The two major isoforms of apoB, apoB-100 and apoB-48, are synthesized by the liver and intestine, respectively. The nature of the lipoprotein particles containing apoB-100 and apoB-48 are of particular interest since they represent particles derived from the liver and intestine and can be used as markers for lipoprotein remnants synthesized by these organs.

Using monoclonal affinity chromatography, lipoprotein particles containing only apoB-100 or apoB-48 were isolated from a patient with apoE deficiency. These results established that the apoB-100 and apoB-48 in the plasma of the apoE-deficient patient were on separate lipoprotein particles, further substantiating the concept that apoB-100 and apoB-48 are secreted on separate lipoprotein particles. A detailed analysis of the lipid composition of these separated apoB-containing lipoprotein particles is currently in progress.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and structural analysis of the plasma apolipoproteins and apolipoprotein isoforms is an ongoing series of studies which are designed to elucidate the physiological role and molecular mechanisms involved in the synthesis, transport, and metabolism of plasma lipoproteins in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of the major plasma and lymph apolipoproteins will be continued, and an evaluation of the presence of plasma precursors of the apolipoproteins will be pursued. The factors which

modulate the extracellular processing of proapoA-I as well as proapoA-II will be evaluated. The covalent structure of variants of the plasma apolipoproteins will continue to be evaluated, particularly with reference to apolipoproteins A-I, A-II, and C-II. The identification of variants which are associated with altered functional activity will provide additional insights into the structural requirements for apolipoprotein function. Studies will also be continued on the molecular defect in Tangier disease including the role of post-translational processing of apoA-I. These studies will add additional insights into the factors modulating the biosynthesis and catabolism of apoA-I and HDL. The continued elucidation of the covalent structure and function of the plasma apolipoproteins will continue to be a prerequisite to our ultimate complete understanding of the molecular mechanisms involved in lipoprotein biosynthesis, transport, catabolism, and atherosclerosis.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02011-09 MDB

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Properties of Lipoproteins and Apolipoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	James C. Osborne, Jr., Ph.D.	Senior Investigator	MDB, NHLBI
Others:	H. Bryan, Brewer, Jr., M.D.	Chief	MDB, NHLBI
	Richard Gregg, M.D.	Senior Investigator	MDB, NHLBI
	Grace Huff, B.S.	Chemist	MDB, NHLBI
	Ellis Kempner, Ph.D.	Senior Investigator	LPB, NIADDK
	Nancy Lee, M.S.	Chemist	MDB, NHLBI
	Ramon R. Tate, Ph.D.,	Senior Investigator	DCRT, NHLBI
	Loren Zech, M.D.	Senior Investigator	OD, NHLBI

COOPERATING UNITS *(if any)*

University of Umea, Sweden (G. Bengtsson, Ph.D. and T. Olivecrona, Ph.D.)

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
7.0	4.0	3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These projects are directed towards a greater understanding of the quaternary organization of plasma lipoproteins and of the function of the oligomeric species involved in the transport and metabolism of lipids in plasma. The apolipoprotein composition of plasma lipoproteins is viewed as the governing factor in directing lipoprotein metabolism. Specificity is believed to be related directly to apolipoprotein secondary, tertiary, and quaternary structure. A knowledge of the equilibrium constants and stoichiometry for the specific complexes formed in plasma by apolipoproteins has allowed us to develop a framework for evaluating the role of apolipoproteins in controlling lipid metabolism. These studies have been extended recently to include lipoprotein lipase and hepatic lipase, two enzymes responsible for triglyceride hydrolysis. We have shown that both active and inactive forms of lipoprotein lipase exist in solution. The active form is the dimer and dissociation results in irreversible inactivation. In contrast, the active form of hepatic lipase, which is much more stable in solution than lipoprotein lipase, is the monomer. Differences in the physical properties and stability of these two enzymes may relate to their role in lipid metabolism *in vivo*.

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Project Description:

Objective:

1) Compartmental modeling of the effect of protein concentration on the rate of inactivation of lipoprotein lipase.

Lipoprotein lipase is the rate-limiting enzyme for the delivery of plasma lipoprotein fatty acids to peripheral tissues. The enzyme is believed to be located at the vascular surface of endothelial cells and catalyzes the release of free fatty acids by hydrolysis of chylomicron and very low density lipoprotein (VLDL) triglycerides. The liberated free fatty acids are taken up by parenchymal cells for immediate oxidation or storage. Although the site of action of lipoprotein lipase is at the capillary endothelium, there is no evidence that the enzyme is synthesized in endothelial cells. Synthesis of lipoprotein lipase is believed to occur in parenchymal cells. Thus, the site of action of the enzyme is distinct from its site of synthesis. Regulation of expressed enzyme activity is therefore a complex interplay between enzyme synthesis and/or activation and secretion by parenchymal cells and enzyme activation/inactivation at the endothelial cell surface. Apolipoprotein C-II, a component of chylomicrons and VLDL, is known to be a specific activator of lipoprotein lipase. The enzyme is known to be unstable in aqueous solution, and enzyme activity at the endothelial cell surface decreases rapidly when delivery of new enzyme is blocked through inhibition of protein synthesis or secretion. We have demonstrated previously, see last year's annual report, that enzyme activity responds dramatically to changes in physical properties, i.e. second and quaternary organization. In addition, we have shown over the past year that the dimer is the active species in solution and that dissociation to monomer is correlated with irreversible loss of enzyme activity (see section 2 of this report). In the present section, we summarize results of compartmental modeling of the rate of loss of enzyme activity as a function of enzyme concentration.

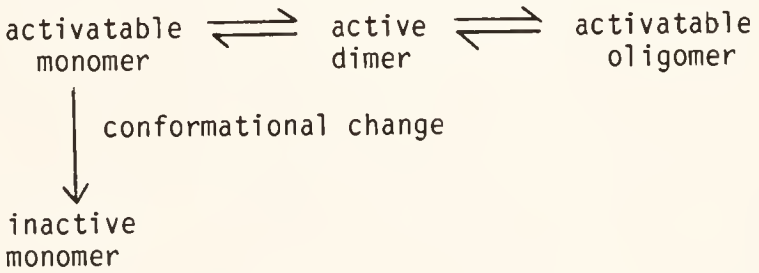
Methods Employed:

The rate of loss of enzyme activity was simulated using the general technique of compartmental analysis, resulting in the formulation of the simplest model which could account for the experimental data. Each compartment in the final model represents a single, homogeneous species and corresponds to a specific molecular species of lipoprotein lipase. The topology of each model is determined by the number of compartments, the connectivity of the compartments, and the enzyme specific activity in each compartment. Given a specific compartmental model, the experimental data were simulated by varying the initial distribution of mass in each compartment along with the rate constants for flow between compartments. Compartmental analysis was carried out using CONSAM, the interactive version of the SAAM simulator on a VAX-11780 Computer (Digital Equipment Corporation).

Major Findings:

We have shown previously that the activity of lipoprotein lipase is stable for long periods in 0.5 M ammonium sulfate, 0.2 M sodium chloride, 10 mM TRIS/Cl, pH 7.4 (10°C) (see last year's annual report). In this buffer, activity is stable

to low levels (< 0.3 M) of guanidinium chloride (GdmCl) and decreases irreversibly over a several hour period at higher (> 0.7 M) concentrations of GdmCl. The rate of loss of activity at 0.75 M GdmCl depended critically upon enzyme concentration but was independent of the presence of other proteins or inactive enzyme protein. Activity loss was measured over a 30-fold (0.01 to 0.32 mg/ml) range in enzyme concentration and analyzed by compartmental modeling. The shape of the inactivation curve depended critically upon the concentration of enzyme; at high concentrations there was little loss of activity during the first hour, whereas at lower concentrations the most rapid loss of activity occurred at early time points. The simplest compartmental model tested which could account for the experimental rates of inactivation at all enzyme concentrations is illustrated below.



Under the framework of this model, dissociation of inactive oligomer results in the formation of additional active dimeric species. This aspect of the model accounts for the initial plateau in stability observed at high concentrations of enzyme. In effect, the presence of inactive oligomers serves as a buffer against the loss of total activity by formation of additional active species through oligomer dissociation with time. At lower concentrations of enzyme, the amount of oligomer is decreased through the laws of mass action, and this results in a more rapid overall rate of inactivation. The relationship between these solution species and the regulation of lipase activity in vivo requires further study. It is tempting, however, to speculate that dimer dissociation may play a role in the rapid changes in lipase activity observed in vivo. Secretion of an enzyme which self-destructs through dimer dissociation would lessen the need for feedback regulation between the parenchymal and nonparenchymal cells of a given tissue. Current studies are directed towards evaluation of the specific rate constants for interconversion of active and inactive species.

Objective:

2) Evaluation of the active species of bovine lipoprotein lipase and human lipoprotein and hepatic lipases.

Human post heparin plasma contains two major lipases, lipoprotein lipase (LPL) and hepatic lipase (HL). LPL is associated with extrahepatic cells and is believed to play a major role in the hydrolysis of triglycerides in chylomicrons and very low density lipoproteins (VLDL). Triglyceride hydrolysis by LPL is increased severalfold in the presence of a specific activator, apolipoprotein C-II (apoC-II). Thus, a deficiency in LPL or apoC-II may lead to the fasting chylomicronemia and mildly elevated VLDL which is characteristic of hypolipoproteinemia. The metabolic role of HL is less well-characterized. This enzyme is associated with hepatic tissue and has been postulated to play a role in high density (HDL) as well as VLDL phospholipid and triglyceride metabolism. The

hydrolysis of triglycerides by HL can be increased severalfold in vitro by apolipoprotein A-II, a major component of HDL. This activation has many of the characteristics of the activation of LPL by apoC-II. Although LPL and HL have many common features, they are clearly different enzymes with distinct enzymatic and physical properties. In order to understand more fully the structure-function interrelationships of these two enzymes, we have measured the active species of human lipoprotein and hepatic lipases and have compared these results with those obtained with the more fully characterized bovine lipoprotein lipase.

Methods Employed:

We have detailed in previous annual reports the development of methodology for measuring the molecular weight of active enzyme molecules in impure or heterogeneous systems. Briefly, the sample is centrifuged to equilibrium in a small fixed angle rotor using a Beckman airfuge at low rotor speeds. The rotor is allowed to stop, and the sample is fractionated from the top of each centrifuge tube using a Hamilton 10 µl syringe. Bovine serum albumin (7.5 mg/ml) added to the sample prior to centrifugation stabilizes the gradient during the fractionation procedure. The activity in each fraction is measured and molecular weights are obtained using distances from the center of rotation for each fraction (calculated from manufacturer's specifications) and the following equation

$$M_w^{app} = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \left(\frac{\partial \ln A}{\partial r^2}\right)$$

where R is the gas constant, T the absolute temperature, ω the angular velocity in radians per second, $\bar{v}$ the partial specific volume, ρ the solvent density, A the activity and r the distance from the center of rotation. Molecular weights obtained in this manner are based on first principles and are independent of shape or composition (except through $\bar{v}$). The drawbacks of the existing method were (1) rotor stability, wobble during deceleration disturbs the sample gradient, and 2) theoretical and practical complexities due to use of a fixed angle rotor. These drawbacks were overcome by use of a swinging bucket rotor (SW50L) and a preparative L2-65B ultracentrifuge. Sample volume was kept to a minimum by use of Teflon inserts in each sample cell.

Major Findings:

Bovine lipoprotein lipase exists in multiple molecular forms in aqueous solution. Sedimentation equilibrium measurements based on total protein demonstrated the presence of reversibly and irreversibly associated species with molecular weights as high as pentameric lipase (M_w^{app} ~ 200,000). Sedimentation equilibrium measurements based on activity were performed at high (~ 0.3 mg/ml) and low (~ 0.01 mg/ml) concentrations of enzyme. In both cases, activity was stable for the duration of the experiment (~ 1 week). The molecular weight at both concentrations of enzyme corresponded to dimeric lipoprotein lipase. Other molecular species in solution were inactive. Human lipoprotein lipase migrated as a protein of molecular weight 55,000 on SDS gel electrophoresis. Sedimentation equilibrium measurements based on activity gave a molecular weight of 105,000 for human lipoprotein lipase. Human hepatic lipase migrates on SDS gel electrophoresis as a protein of molecular weight 53,000. The active species of

this enzyme corresponds to the monomer (M_W^{app} = 54,000). Thus, bovine and human lipoprotein lipase are active as the dimeric species and inactive in other molecular forms. Results from other studies indicate that dimer dissociation causes irreversible inactivation. Human hepatic lipase, which is much more stable than human or bovine lipoprotein lipase, is active in the monomeric form. The differences in molecular forms and stability observed in vitro may relate to differences in the metabolic function of these enzymes in vivo.

Objective:

3) Evaluation of the molecular weight of bovine lipoprotein lipase by radiation inactivation.

The monomeric molecular weight of bovine lipoprotein lipase in denaturant as measured by equilibrium sedimentation (see last year's annual report) is 41,700. This value does not depend upon shape or carbohydrate content and as expected is much lower than the value of 60,000 estimated by gel electrophoresis. In the absence of denaturant, oligomers as high as octamers of bovine LDL exist in solution. Most of these species are, however, inactive. The active species of enzyme, as determined by sedimentation equilibrium using activity measurements, is the dimer (see section 2 of this report). The concentration oligomers in solution increase with time and this transition is paralleled by an irreversible loss of enzyme activity. The rate of this process can be controlled with buffer composition and is dependent upon the concentration of active enzyme; the rate of inactivation increases with decreasing concentration of enzyme (i.e. dimer dissociation) and is independent of the concentration of inactive enzyme protein. Inactivation at all enzyme concentrations is correlated with a conformational change in the enzyme molecule. Thus, it appears that dimer dissociation results in an irreversible refolding of the monomer to an inactive state. In this section we summarize results of our studies on the minimal functional unit required for enzyme activity as measured by radiation inactivation.

Methods Employed:

The assumption used in the estimates of molecular weight by radiation inactivation are:

1) Primary ionizations are due to collisions between accelerated electrons and the macromolecule.

2) Primary ionizations are distributed randomly throughout the sample volume.

3) There are no indirect ionizations due to diffusion of free radicals generated from primary ionizations.

4) A single primary ionization (equivalent to the release of ~ 1500 Kcal/mole in energy) is sufficient to disrupt an entire polypeptide chain.

5) There is no energy transfer between noncovalently bound protomers in a macromolecular complex.

With these assumptions target theory predicts that inactivation obeys the following equation:

$$A(D)/A_0 = e^{-KD}$$

where

A_0 = original activity
D = radiation dose
A(D) = activity remaining after dose D
K = effective target volume

The effective target volume is obtained from the slope of a plot of ln A/A_0 versus D (rads), and the molecular weight is obtained from a knowledge of the sample density (ρ) and Avogadro's number ($\mathcal{N}$).

$$M = \rho \mathcal{N} K$$

Major Findings:

A linear electron accelerator producing 13 MeV electrons at the Armed Forces Radiobiology Research Institute was used for radiation exposure. In order to minimize indirect effects, due to diffusion of free radicals from the location of the primary ionization, the samples were irradiated at -135°C. The buffer used was 0.5 M ammonium sulfate, 0.2 M sodium chloride, 0.001 M sodium azide, 0.01 M TRIS/Cl, pH 7.4, which contained 5 mg/ml bovine serum albumin. As a control enzyme, glucose-6-phosphate dehydrogenase (0.3 unit/ml) was also included in each sample. The samples were sealed in 2-ml glass vials after previous rapid freezing in a slurry of dry ice and ethanol. After irradiation, the vials were opened and the samples were analyzed for lipoprotein lipase and glucose-6-phosphate dehydrogenase activity. Plots of ln A/A_0 versus radiation dose were linear for both enzymes. Molecular weights were calculated as indicated in the Methods Employed section above. Glucose-6-phosphate dehydrogenase gave the expected molecular weight of 104,000. Bovine lipoprotein lipase was analyzed at low (0.01 mg/ml) and high (0.3 mg/ml) concentrations of enzyme in order to detect effects of dimer dissociation on the minimal functional unit required for activity. The molecular weight at low and high concentrations of enzyme was 72,000. Radiation inactivation of glycoproteins results in molecular weights that correspond only to the polypeptide portion of the macromolecule. Presumably this is due to the inability of a primary ionization in the carbohydrate moiety to disrupt the polypeptide backbone of enzymes. Bovine lipoprotein lipase contains 8.3% carbohydrate. Therefore, the 72,000 species observed by radiation inactivation corresponds to the dimer. We have shown previously by sedimentation equilibrium that the dimer is the active species in aqueous solutions of bovine lipoprotein lipase and that dimer dissociation results ultimately in irreversible inactivation. These studies do not, however, rule out the possibility that monomers may exhibit activity. The results of radiation inactivation are consistent with the concept that the dimer is the minimal molecular unit required for active bovine lipoprotein lipase.

Significance to Biomedical Research and the Program of the Institute: These investigations are directed towards a greater understanding of the quaternary

organization and function of the plasma lipoproteins. The apolipoprotein and lipid composition of plasma lipoproteins is related directly to the concentration and composition of other components of plasma, including other plasma lipoproteins. Enzymic hydrolysis of lipids in plasma is mediated through apolipoprotein effectors which distribute among plasma lipoproteins according to the laws of mass action. A quantitative knowledge of these types of interactions is fundamental to our understanding of lipid transport and metabolism in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course: Investigations concerning the molecular properties of apolipoproteins shall be continued. A major emphasis shall be the purification of human lipoprotein lipase and hepatic lipase and the production of specific antibodies for these enzymes. These studies continue to form a framework for our ultimate understanding of in vivo plasma lipoprotein interactions.

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11. Kincaid, R.L., Manganiello, V.C., Odya, C.E., Osborne, J.C., Jr., Stith-Coleman, I.E., Danello, M.A., and Vaughan, M.: Purification and properties of calmodulin-stimulated phosphodiesterase from mammalian brain. J. Biol. Chem. 259: 5158-5166, 1984.

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13. Osborne, J.C., Jr.: Delipidation of plasma lipoproteins. Meth. Enzymol., in press.

14. Osborne, J.C., Jr., Lee, N.S., and Huff, G.M.: Solution properties of plasma lipoproteins. Meth. Enzymol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02012-09 MDB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Z.H. Beg	Research Chemist	MDB, NHLBI
Others:			
	J.A. Stonik	Chemist	MDB, NHLBI
	H.B. Brewer, Jr.	Chief	MDB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously reported that the enzymic activity of rat and human liver 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR) is modulated in vitro in a bicyclic cascade system involving reversible phosphorylation of HMGR and reductase kinase.

A calcium-activated and phospholipid-dependent protein kinase (protein kinase C) purified from rat brain cytosol is able to phosphorylate both insoluble microsomal (MW ~ 100,000) and purified soluble (MW 53,000) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). The inhibition of enzymic activity and phosphorylation of HMGR was maximal at pH 6.5.. Lysine rich histone (type III-S) was the preferred substrate for protein kinase C. The phosphorylation of mixed casein was significantly increased by protein kinase C. The phosphorylation of phosvitin and protamine was not significantly stimulated by phospholipid and diolein. The phosphorylation and concomittant inactivation of enzymic activity of HMGR was absolutely dependent on calcium, phosphatidyl serine and diolein. Dephosphorylation of phosphorylated HMGR was associated with the loss of protein bound radioactivity and reactivation of enzymic activity. Maximal phosphorylation of purified HMGR was associated with the incorporation of 1.05 ± .016 mol of phosphate per mol of native form of HMGR (MW ~ 100,000). The apparent Km for purified HMGR and histone was .08 mg/ml, and .12 mg/ml, respectively. The tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) stimulated the protein kinase C catalyzed phosphorylation of HMGR. Increased phosphorylation of HMGR by PMA suggests a possible *in vivo* protein kinase C mediated mechanism for the short-term regulation of HMGR activity.

The identification of the protein kinase C system in addition to the reductase kinase-reductase kinase kinase bicyclic cascade system for the modulation of the enzymic activity of HMGR may provide new insights into the molecular mechanisms involved in the regulation of cholesterol biosynthesis.

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Project Description:

Objectives: The current research of this laboratory is related to the identication of a new protein kinase, protein kinase C, in addition to reductase kinase, which modulates the of enzymic activity of HMGR.

Methods Employed:

i) Purification of Protein Kinase C from Rat Brain:

Protein kinase C was partially purified from the soluble fraction of fresh rat brains essentially by the method of Kikkawa et al (J. Biol. Chem. 257, 13341-13348, 1982). Fresh brains from rats were removed and washed in ice-cold buffer A (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, .5 mM PMSF, .1 mM TLCK, .1 mM TPCK, 50 µM leupeptin and 50 mM 2-mercaptoethanol). The cerebra (~ 23 g) were homogenized in 6 vol of buffer A in a glass-Telflon pestle homogenizer and the homogenate centrifuged at 16,000 xg for 10 min. The supernatant was recentrifuged at 340,000 xg for 30 min. The supernatant (100 ml) was applied to a DEAE-cellulose (DE-52) column (2.6 x 9.3 cm; flow rate 50 ml/hr) equilibrated with buffer A containing 10% glycerol (buffer B). After washing the column with 10 ml of buffer B, the column was washed with 500 ml of buffer C (20 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, .5 mM PMSF, .1 mM TPCK, .1 mM TLCK, 50 µM leupeptin and 10% glycerol). Protein kinase C was eluted with a gradient of buffer C (300 ml) and buffer C containing .3 M NaCl (300 ml). Nine ml fractions were collected and assayed for protein kinase C activity. Fractions containing Ca^{++} and phospholipid dependent histone kinase activity were pooled and fractionated with 0-60% ammonium sulfate. The precipitate was dissolved in buffer C (vol. 5.1 ml) and 5 ml was applied to a Sephadex G-150 column (2.6 x 85 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 0.5 mM EGTA, .5 mM EDTA and 10% glycerol. Five ml fractions were collected and assayed for protein kinase C activity. Protein kinase C was stable for months when stored in 30% glycerol at -70°C. The purified enzyme had a specific activity of 65 nmole of ^{32}P transferred/min/mg protein (average of six determinations of different fractions) when lysine-rich histone H_1 was used as a substrate. This preparation was free from cAMP dependent protein kinase activity, reductase kinase actiivty and was totally dependent on Ca^{++} and phospholipid for protein kinase C activity.

ii) Isolation and Assay of Microsomal and Purified HMGR Activity:

Microsomes isolated from livers of rats fed 3% cholestyramine for 5 days and killed at the peak of diurnal rhythm were used for solubilization and purification of homogeneous HMGR. This method of isolation involves the purification of the 53,000 molecular weight form of HMGR following a protease mediated solubilization of microsomal HMGR. Antibodies to purified rat liver HMGR were prepared in New Zealand rabbits by subcutaneous injections of a total of 480 µg of protein per rabbit. The first two injections (1st and 3rd weeks) were in complete Freund's adjuvant; all subsequent injections were given at 12 and 17 week intervals in incomplete Freund's adjuvant. Blood was withdrawn 18,

19, 20, and 21 weeks following the initial injection and analyzed for antibody titer. The native form of microsomal HMGR (M_r ~ 100,000) was isolated and the microsomal pellet containing native insoluble membrane bound HMGR was suspended in 40 mM MOPS, (pH 6.5), 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 50 µM PMSF, 50 µM leupeptin and 10% glycerol (buffer A). The microsomal suspension was heated at 50°C for 15 min. This technique selectively inactivates the heat labile microsomal reductase kinase whereas HMGR activity remains unaffected. An aliquot of heat treated microsomal HMGR was solubilized by homogenizing in 1% lubrol followed by centrifugation (100,000 xg, 60 min). The supernatant was fractionated with 60% ammonium sulfate and the precipitate dissolved as well as desalted in buffer A. Microsomal and solubilized HMGR were utilized for the inactivation and phosphorylation of the native high molecular form (~ 100,000) of HMGR with protein kinase C. Enzymic activity of microsomal, solubilized or purified HMGR was determined as previously described.

iii) Assay of Protein Kinase C Activity and Protein Phosphorylation:

The reaction mixture in a total volume of 50 µl contained either 30 mM Tris-HCl (pH 7.5) or MOPS (pH 6.5), 6 mM magnesium acetate, .6 mM $CaCl_2$, 20 µg/ml phosphatidylserine, 4 µg/ml diolein, 1-2 mg/ml of lysine-rich histones (type III-S), .1 mg/ml of HMG-CoA reductase (unless stated otherwise), .05 mM [γ-^{32}P]ATP (specific actiivty, 1,700 - 3,500 cpm/pmol), and the protein C kinase fraction to be assayed. Phospholipids and diolein were mixed together in chloroform, dried by a stream of N_2 gas, and the residue suspended in 20 mM Tris-HCl (pH 7.5) by sonication (Branson, Model W 185) at 40 W for 5 min at 0°C. The reaction was initiated by the addition of radiolabeled ATP and performed at 30°C for 15 -30 min. The reaction was terminated by pipetting 10 µl of the reaction mixture in duplicate onto Whatman 3 mM filter discs which had earlier been spotted with 40 µl of 20% trichloroacetic acid containing 5 mM tetrasodium pyrophosphate and 1 mM ATP. The filter discs were then washed, dried and assayed for radioactivity in 10 ml of hydrofluor. One unit of protein kinase C is defined as the activity which transfers 1 nmol of phosphate from radiolabeled ATP to histone (type III-S) per min under the assay conditions described above.

For polyacrylamide gel electorphoresis, aliquots were added to tubes containing 25 µl of 1% $NaDodSO_4$, 2 mm DTT, 30% glycerol, .002% bromophenol blue and heated in boiling water for 4 min. The following marker protein was used: myosin (M_r 205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (68,000), and ovalbumin (45,000). In phosphorylation studies, gels were stained, destained, and then dried between two sheets of cellophane paper. The dried gels were exposed to an x-ray film at -70°C to prepare the autoradiograph.

In experiments involving immunoblotting, proteins were electorphoretically transferred from the slab gels to nitrocellulose paper in 25 mM Tris, 190 mM glycine (pH 8.3) and 20% methanol. The paper was incubated for 60 min. in 3% gelatin containing 500 mM NaCl and 20 mM Tris-HCl (pH 7.5) with gentle agitation. The paper was then incubated with antireductase antiserum (25 µl/ml) and three washes as described in the Bio-Rad immunoblotting (GAR-HRP) assay kit instructions. The paper was finally incubated with horseradish peroxidase conjugated goat anti-rabbit IgG, followed by three washings and a 20 min incubation with peroxidase color development reagent.

Major Findings:

Effect of pH on Enzymic Activity: The effect of pH on the activity of protein kinase C was examined. The enzyme had a broad pH optimum with a maximum at approxiamtely pH 7.5 when histone H_1 was used as a substrate. However, both the inhibition of enzymic activity and phosphorylation of HMGR was maximal at pH 6.4-6.5. Accordingly, in all the experiments involving HMGR reported here, 30 mM MOPS at pH 6.5 was employed instead of the pH 7.5 30 mM Tris-HCl buffer.

Substrate Specificity: The activity of protein kinase C was assayed utilizing different substrates including HMGR. The assays were performed in the presence of Ca^{++}, with and without phosphatidyl serine and diolein. Lysine rich histone H_1 (type III-S) was the preferred substrate for protein kinase C. Histone VII-S is slightly lysine rich and was not significantly phosphorylated. The phosphorylation of mixed casein was significantly increased by protein kinase C. Phosphorylation of mixed casein was not stimulated by phospholipid and diolein. Protein kinase C was able to catalyze a phospholipid dependent phosphorylation of rat liver HMGR. Due to the absence of a known physiological substrate of protein kinase C, we have compared the kinetics of HMGR phosphorylation with histone H_1, the most preferred and widely used substrate. The K_m values for HMGR and histone H_1 were .08 mg/ml, and .12 mg/ml, respectively.

Inactivation and Reactivation of HMGR: A time dependent inactivation of purified HMGR in the presence of ATP-Mg, protein kinase C, Ca^{++} and phospholipid was obtained. The inactivation of HMGR was dependent on protein kinase C, and ATP-Mg concentration in the presence of optimal levels of Ca^{++} (.5 mM) and phospholipids (20 µg/ml). Incubation of inactivated HMGR with partially purified hepatic phosphoprotein phosphatase was associated with a time-dependent reactivation (dephosphorylation) of the enzymic activity of HMGR. The reactivation by phosphatase was completely blocked by NaF.

Protein Kinase C Dependent Inactivation (Phosphorylation) of Purified and Microsomal HMGR: The enzymic activity of purified HMGR is maximally inhibited only when both Ca^{++} and phospholipid are present with protein kinase C. No loss in activity was observed in the absence of ATP-Mg, protein kinase C, or the presence of protein kinase C without Ca^{++} and phospholipid. ADP alone or in combination with ATP failed to decrease HMGR activity. The inhibition of purified HMGR by the Ca^{++}, phospholipid dependent protein kinase C was not increased by either cAMP or cGMP.

The enzymic activity of HMGR in microsomal preparations containing either protease cleaved (M_r 53,000) or the native form (M_r ~ 100,000) was inhibited by ATP-Mg in the presence of protein kinase C, Ca^{++}, and phospholipids. Native HMGR when solubilized by lubrol was equally susceptable to inactivation by protein kinase C mediated phosphorylation. The inactivated (phosphorylation) HMGR from microsomal preparations containing the ~ 100,000 molecular form was also reactivated (dephosphorylated) by phosphoprotein phosphatase.

Phosphorylation and Dephosphorylation of HMGR: The time course of phosphorylation of purified rat hepatic HMGR by protein kinase C revealed increasing incorporation of radiolabeled phosphate with increasing time. Autoradiogram of the samples at different time intervals following $NaDodSO_4$ gel electrophoresis revealed that [^{32}P]-phosphate incorporation had occurred only in the 53,000 subunit of HMGR. HMGR was not phosphorylated by protein kinase C when Ca^{++} and phospholipid in the absence of HMGR did not contain radioactivity in the position of the 53,000 subunit of HMGR. Dephosphorylation of [^{32}P]-HMGR was associated with a time-dependent release of radioactivity. The autoradiogram of phosphorylated and dephosphorylated HMGR, revealed a virtual total loss of radioactivity in HMGR following a 7.5 min incubation of phosphorylated HMGR with phosphoprotein phosphatase.

Protein kinase C-mediated phosphorylation of the native form of microsomal HMGR (M_r ~ 100,000) was also observed. In the presence of Ca^{++}, and phospholipids, protein kinase C, catalyzed the phosphorylation of lubrol-solubilized microsomal HMGR which was analyzed by immunoblotting following $NaDodSO_4$ gel electrophoresis and electrophoretic transfer to nitrocellulose paper. The electrophoretic mobility of the native subunit of HMGR corresponded to an apparent molecular weight of ~ 100,000. The autoradiogram of the immunoblott confirmed the identity of the ~ 100,000 molecular form of HMGR. Autoradiogram of the immunoblott from control samples (-PL, -Ca^{++}, + 10 mM EGTA) did not reveal significant radioactivity in the position of the ~ 100,000 subunit of native HMGR.

When purified rat liver HMGR (.1-.4 mg/ml) was incubated for 60-120 min with protein kinase C under standard assay conditions, 1.05 ± .016 mol of phosphate were maximally incorporated per mol of HMGR (M_r ~ 100,000) in seven separate experiments involving six different preparations of homogeneous HMGR.

Effect of PMA on Protein Kinase C Activity: The phosphorylation of HMGR with protein kinase C and phosphatidyl serine was demonstrated in the presence of varying concentrations of Ca++ and fixed levels of EDTA, EGTA, and ATP-Mg. The tumor-promoting phorbol ester, PMA significantly increased the protein kinase C catalyzed phosphorylation of HMGR at substurating concentration of Ca^{++} and fixed concentration of PMA (15 ng/ml).

Significance to Biomedical Research and the Program of the Institute:

The results detailed here are of considerable importance since the protein kinase C mediated phosphorylation of HMGR would be anticipated to have specific physiological, i.e., inhibition of HMGR activity and inhibition of cholesterol biosynthesis. Based on these results and our previous studies we now propose that the enzymic activity of HMGR is modulated by two kinase systems. The first system involves the bicyclic cascade system utilizing reductase kinase and reductase kinase kinase, while the second system involves protein kinase C. Increased phosphorylation and inhibition of HMGR enzymic activity by phorbol esters suggests that the protein kinase C mediated phosphorylation of HMGR may be an important *in vivo* mechanism. The elucidation of the modes of control of HMG-CoA reductase will allow a detailed analysis of the parameters involved in the cellular regulation of cholesterol metabolism in normal sujects and patients with atherosclerosis.

Proposed Course:

A systematic investigation of the second kinase system will be continued. Possible in vivo role of phorbol ester will also be investigated. Attempt will be made to determine which physiological systems regulate the enzymic activity of HMGR by modulating the activity of the two separate protein kinase systems.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02019-06 MDB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of Lipoproteins and Apolipoproteins in Humans

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6	4	2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A technique necessary for apolipoprotein metabolic studies is to quantitate apolipoproteins. We developed an ELISA assay for apoB and are presently developing ones for apoA-I, A-II, C-II, and E. ApoE binds to the LDL receptor in vitro. In vivo studies of apoE were performed in subjects who lack the LDL receptor. The fractional catabolic rate (FCR) of apoE was normal indicating the LDL receptor is unlikely to be an important site of catabolism for apoE. Two forms of apoE, apoE2 and apoE2*, associated with type III hyperlipoproteinemia (HLP) were evaluated by in vivo kinetic studies. The metabolism of apoE2 and apoE2* were the same and the FCR for both significantly slower than for normal apoE. The metabolism of apoA-I has been investigated in a number subjects. In type V HLP subjects, who have decreased apoA-I levels, the FCR of apoA-I was increased with a normal synthesis rate. Tangier disease subjects have decreased levels of apoA-I with a relative increase of proapoA-I. The rate of conversion of proapoA-I to mature apoA-I was normal and the relative increase in proapoA-I was due to rapid catabolism of mature apoA-I. A genetic variant of apoA-I with one additional unit of negative charge was investigated and found to have a normal FCR. There are two forms of apoB in plasma, apoB-48 of intestinal origin and apoB-100 of hepatic origin. Their metabolism was investigated in subjects lacking the LDL receptor. VLDL apoB-48 was catabolized at a normal rate with little conversion to IDL and LDL, similar to normal subjects. VLDL apoB-100 had a slower FCR with increased conversion to IDL and LDL compared to normals. Therefore, the LDL receptor does not have a role in apoB-48 metabolism but does regulate apoB-100 metabolism. Subjects homozygous for apoE2 have decreased LDL and apoB concentrations. LDL apoB from these subjects was catabolized at a decreased FCR compared to normal LDL apoB. Also, these subjects catabolized both normal and their own LDL apoB more rapidly than normals. The decreased LDL apoB levels were due to both a decreased production rate and an increased FCR. Therefore apoE has complex effects on LDL apoB metabolism.

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Project Description

The current research of this laboratory is the following:

Objective:

1) To develop the methodology for the measurement of apolipoproteins by enzyme linked immunoassay techniques.

Methods Employed:

In a competitive solid phase enzyme linked immunosorbent assay the antigen is bound to polystyrene microtite plate wells followed by the addition sample or standard and an antibody against the substance to be quantitated. The wells are washed and the amount of antibody bound to the plates is assayed using an alkaline phosphatase conjugated anti-IgG antibody. From this, the concentration of analyte in the assay sample can be determined.

Major Findings:

A solid phase enzyme linked immunosorbent assay for apoB has been developed in the Branch. In this competitive assay, LDL is first bound to the polystyrene microtiter plate wells followed by washing with an albumin solution. The sample to be assayed and anti-apoB mouse monoclonal antibody is next added to the plate. After incubation the unbound antibody is washed from the plate and an anti-mouse IgG antibody coupled with alkaline phosphatase is added. Unbound antibody is washed from the plate and substrate for alkaline phosphatase is added. Color developement is determined spectrophotometrically and the apoB concentration determined. This assay is able to quantify apoB in all lipoprotein classes equally, is able to quantify as little as 1 ng of protein, and is very reproducable (coeffiecient of variation = 6%). It also has the advantage of using entirely commercially available reagents and being able to be automated using equipment designed for microtiter plates. One person should be able to assay 1000 samples a week using this assay when it is automated. Enzyme linked immunosorbent assays for apoA-I, apoA-II, apoC-II, and apoE are presently being developed.

Objective:

2) To study the metabolism of apolipoprotein E in normal and dyslipoproteinemic subjects.

Methods Employed:

ApoE kinetics were studied by isolating apoE by ultracentrifugation and column chromatography, radioiodinating with iodine monochloride, reassociating it with lipoproteins, and injecting it intravenously into the study subjects. Multiple timed plasma samples were obtained, the lipoproteins separated by

ultracentrifugation, and the rate of catabolism determined from the radioactive decay curve by computer assisted multiexponential curve fitting.

Major Findings:

ApoE is found in plasma primarily on VLDL and HDL and is important in modulating the catabolism of remnants of triglyceride rich lipoprotein particles. It has been shown by other investigators that apoE binds to two distinct receptors in in vitro receptor binding studies. One of these receptors is specific for apoE, the apoE receptor; while the other binds both apoE and apoB on LDL; the apoB,E or LDL receptor. To investigate the importance of each of these receptors in the metabolism of apoE in vivo, the kinetcs of apoE metabolism were determined in subjects with homozygous familial hypercholesterolemia who lack a functional apoB,E receptor, and in normal subjects. The residence time of apoE was normal in the subjects lacking the apoB,E receptor indicating that the binding of apoE to the apoB,E receptor is unlikely to be important in the in vivo catabolism of apoE. In addition, the apoE concentration in plasma was 2 to 3 fold elevated in the homozygous familial hypercholesterolemia subjects and this elevation was due to an increased production rate of apoE.

ApoE is a polymorphic protein with 3 common alleles being present in the normal population and these alleles are inherited in a co-dominant fashion at a single genetic locus. Most normal subjects are homozygous for the E^3 allele while type III hyperlipoproteinemia is associated with homozygosity for the E^2 allele. The $apoE_2$ protein differs from $apoE_3$ at amino acid 158 with $apoE_2$ having a cycteine instead of arginine. Recently, there has been described an individual with type III hyperlipoproteinemia whose apoE, designated $apoE_2$*, differs from normal $apoE_3$ at amino acid position 145 with a cysteine being substituted for an arginine. In addition, $apoE_2$* has approximately 50% of normal binding activity to fibroblasts while $apoE_2$ has less than 1% of normal binding activity. We have previously shown that $apoE_2$ is catabolized in vivo more slowly that $apoE_3$, and in collaboration with Dr. Richard Havel, we have performed similar in vivo studies utilizing $apoE_2$*. $ApoE_2$* was catabolized at the same fractional catabolic rate as $apoE_2$ and both of these were catabolized at a slower rate than $apoE_3$ in both normal and type III hyperlipoproteinemia subjects. Therefore kinetically $apoE_2$ and $apoE_2$* were indistinguishable and these results are 1) consistant with the finding that the subjects with both types of apoE have type III hyperlipoproteinemia and 2) are at variance with the in vitro binding data as to the relative degree of abnormality of $apoE_2$ and $apoE_2$*.

Objective:

3) To study the metabolism of normal and variant forms of apolipoprotein A-I in normal and dyslipoproteinemic subjects.

Methods Employed:

The methods for performing apoA-I kinetic studies have been modified and extended. ApoA-I and isoforms of apoA-I were isolated from plasma and lymph by ultracentrifugation, column chromatography, and preparative sodium dodecyl sulfate and isoelectrofocusing gel electrophoresis. The isolated isoforms of

apoA-I were iodinated by the iodine monochloride method, injected into study subjects, and multiple timed samples of plasma obtained. The radiolabeled isoforms of apoA-I were then isolated by ultracentrifugation and preparative isoelectrofocusing; and the rate of catabolism of radiolabeled apoA-I isoforms from plasma was determined by computer curve fitting.

Major Findings:

ApoA-I is synthesized as a preproprotein. There is a co-translational cleavage of the pre-peptide and the proapoA-I is secreted into plasma. In normal subjects there is a rapid quantitative conversion of proapoA-I to mature apoA-I with little direct catabolism of proapoA-I. Individuals with type V hyperlipoproteinemia have decreased plasma concentrations of HDL cholesterol and apoA-I. In order to evaluate the kinetic cause of the decreased apoA-I concentrations and to determine if the rate of the conversion of proapoA-I to mature apoA-I had an important regulatory function in setting the plasma apoA-I concentration, the kinetics of apoA-I metabolism in type V subjects was studied. In these subjects apoA-I was catabolized at an increased fractional catabolic rate and they had a normal production rate. In addition, there was a normal rate of conversion of proapoA-I to mature apoA-I in these patients. In type V hyperlipoproteinemia, the decreased plasma apoA-I concentrations are due to an increased fractional catabolic rate and the rate of conversion of proapoA-I to mature apoA-I did not play an important part in modulating the catabolic rate of apoA-I.

Tangier disease is characterized by very low concentrations of plasma HDL and apoA-I and apoA-II. On isoelectrofocusing, the predominant forms of apoA-I in Tangier disease are proapoA-I and mature apoA-I while in normal subjects mature apoA-I is the predominate form with very small amounts of proapoA-I. These findings have prompted other investigators to hypothesize that the primary defect in Tangier disease is an inability to convert proapoA-I to mature apoA-I. An alternative hypothesis is that mature apoA-I from Tangier subjects is catabolized very rapidly and the reason the proapoA-I is so prominent is a relative deficiency of mature apoA-I rather than an enrichment of proapoA-I. To test this hypothesis, proapoA-I from normal and Tangier disease subjects were isolated, iodinated,and injected into normal subjects and patients with Tangier disease. Both normal and Tangier proapoA-I were rapidly converted to mature apoA-I in both normal and Tangier disease subjects. There is no significant defect in the conversion of Tangier proapoA-I to mature apoA-I or a defect in the conversion of normal proapoA-I to mature apoA-I in patients with Tangier disease. Therefore, the relative increase in proapoA-I in subjects with Tangier disease is due to rapid catabolism of mature apoA-I in these subjects and is not due to a defect in the conversion of pro to mature apoA-I.

Dr. Gert Utermann from Marburg, Germany has identified a family with an apoA-I variant, designated apoA-$I_{Marburg}$. The subject has normal lipid and lipoprotein concentrations in plasma but has two major isoforms of apoA-I on isoelectrofocusing, one at the normal mature apoA-I position and one with an additional negaive unit of of charge. Both of these isoforms were isolated from this subject as was mature apoA-I from a normal subject. These isoforms of apoA-I were iodinated and injected into three normal subjects. All three isoforms were catabolized at virtually identical rates with no rapid conversions between isoforms. These results are consistent with the affected subject being heterozygous with one normal and one abnormal allele for apoA-I, the abnormal allele has a single point mutation coding for a protein with one negative charge

unit difference from normal mature apoA-I and this mutation does not significantly alter apolipoprotein or lipoprotein metabolism.

Objective:

4) To study the metabolism of LDL and apolipoproteins B-100 and B-48 in normal and dyslipoproteinemic subjects.

Methods Employed:

The kinetics of apoB-100 and apoB-48 were studied using isolated lipoproteins. VLDL isolated by ultracentrifugation from a patient with apoE absence was used as the source of apoB-48 and VLDL apoB-100. LDL from normals and subjects homozygous for $apoE_2$ was used to study LDL apoB-100 kinetics. The isolated lipoproteins were iodinated by the iodine monochloride method, injected into the study subjects, and multiple timed samples of plasma obtained. The different lipoprotein subclasses were then isolated by ultracentrifugation. ApoB-100 and apoB-48 were separated utilizing sodium dodecyl sulfate gel electrophoresis with slab gels consisting of a mixture of polyacrylamide and agarose. The plasma catabolism of apoB-100 and apoB-48 was evaluated and the residence times and multicompartmental modelling of the apoB isoforms was determined using the SAAM 27 computer program.

Major Findings

Human plasma apoB exists in two major forms designated apoB-48 and apoB-100. ApoB-48 is of intestinal origin while apoB-100 is of hepatic origin. ApoB-100 is the recognition site on LDL for binding to the high affinity LDL receptor. The defect in homozygous familial hypercholesterolemia (FH) is a defective or absent high affinity LDL receptor, and LDL apoB-100 is catabolized slowly compared to normals. To determine if the LDL receptor is important in the metabolism of apoB-48 containing lipoproteins and in the conversion of hepatic VLDL to IDL and LDL, the catabolism of VLDL apoB-48 and apoB-100 in FH homozygotes was analyzed. In normal sujects apoB-48 was rapidly catabolized from VLDL and less than 10% of the apoB-48 was converted to IDL and LDL. Very similar results were obtained in FH patients with rapid catabolism of apoB-48 from VLDL and little conversion of IDL and LDL. In homozygous FH patients, VLDL apoB-100 had a prolonged residence time. In addition 50% of it was converted to IDL and LDL with 50% direct catabolism while only 15% was converted in normal subjects with 85% direct catabolism. These results indicate 1) intestinal apoB-48 lipoprotein metabolism is normal in FH homozygotes and the LDL receptor is not required for catabolism of apoB-48 in vivo; and 2) that there is direct catabolism of VLDL from plasma in normal subjects via the LDL receptor, and in the absence of the LDL receptor the fraction of VLDL converted into IDL and LDL is increased.

Subjects with type III hyperlipoproteinemia are homozygous for $apoE_2$ and usually have reduced plasma LDL cholesterol and apoB concentrations. In addition, there are individuals who are normolipidemic and homozygous for $apoE_2$. These individuals also have reduced plasma LDL cholesterol and apoB concentrations. It is commonly felt that the function of apoE is to modulate the catabolism of remnants of triglyceride-rich lipoproteins with no important

role in LDL metabolism, but because of the low LDL apoB concentrations it was of importance to analyze the kinetics of LDL metabolism in these subjects.

LDL was isolated from normolipidemia or hyperlipidemic $apoE_2$ and normolipidemic $apoE_3$ homozygous subjects, radiolabeled, and both types of LDL were injected into $apoE_2$ and $apoE_3$ homozygotes. The $apoE_2$ subjects had LDL apoB concentrations that were markedly decreased. The LDL from the $apoE_2$ subjects was catabolized more slowly than the LDL isolated from $apoE_3$ subjects in both types of subjects. The $apoE_2$ subjects catabolized both forms of LDL faster than the $apoE_3$ subjects. Comparing the metabolism of LDL isolated from $apoE_3$ subjects in $apoE_3$ subjects, the LDL prepared from $apoE_2$ subjects and analyzed in $apoE_2$ subjects had a significantly greater fractional catabolic rate with a marked reduction in production rate. Several conclusions can be drawn from these studies: 1) there is an unpregulation in LDL catabolism in $apoE_2$ subjects; 2) these subjects produce an LDL that is catabolized abnormally slow; 3) the low LDL apoB concentration in $apoE_2$ subjects is due both to decreased production and rapid catabolism of LDL; and 4) structural alterations in apoE have important effects on LDL metabolism.

Significance to the Biomedical Research Program of the Institute

Lipoproteins are very important in initiating and modulating the atherosclerotic process. Apolipoproteins are central to the control of lipid and lipoprotein metabolism and by understanding the metabolism of apolipoproteins new insights are gained into the control of the development of atherosclerotic vascular disease. There are a number of dyslipoproteinemic states in which there is a known abnormality in an apolipoprotein or apolipoprotein receptor. These include type III hyperlipoproteinemia, apoE absence, familial hypercholesterolemia and Tangier disease. By studying these dyslipoproteinemic states in which nature has introduced a known specific pertubation, one can gain a more complete understanding of lipoprotein metabolism in these patients as well as normal subjects. This will allow a more complete and rational approach to the therapy of diseases characterized by dyslipoproteinemia.

Proposed Course:

It is proposed to extend these studies in the following ways:

1) The development of an enzyme linked immunoassay for apoA-I will be completed. Eventually the immunoassays for other apolipoproteins will also be converted to the enzyme linked immunoassay method because of inherent advantages of this type of assay compared to the present methods being employed.

2) Additional apoE metabolic studies will be performed. The metabolism of $apoE_2$ will be studied after the reactive sulfhydryl group on its two cyteine residues have been modified by a neutral reagent and by reagents that will introduce a positive or negative charge at these sites. In addition, the metabolism of new mutant forms of apoE ($apoE_1$ and $apoE_2$) will be investigated. Collaborative studies will be initiated to study the in vitro binding of apoE to cellular membranes and to evaluate the affect of apoE on modulating lipoprotein lipase and hepatic lipase.

3) ApoA-I and apoA-II studies will continue. The isoforms of apoA-II will be isolated, characterized, and kinetic studies will be performed as have

previously been carried out with apoA-I. In addition, other genetic variants of apoA-I and apoA-II will be isolated and the kinetics of their metabolism will be determined.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02022-04 MDB

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular Lipid and Lipoprotein Biochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jeffrey M. Hoeg, M.D.	Medical Staff Fellow	MDB, NHLBI
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	Steven Merlin	Chemist	MDB, NHLBI
	Gilbert Olsen	Chemist	MDB, NHLBI
	Briston Williamson	Lab. Tech.	MDB, NHLBI
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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.2	1.2	4.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The primary focus of this laboratory is to evaluate, characterize, and quantitate the interaction of specific lipoproteins and apolipoproteins with certain cell types. By studying the effects of the lipoproteins on the lipid and lipoprotein metabolism of fibroblasts, macrophages, and hepatocytes from normal and dyslipidemic subjects, an understanding of normal lipoprotein physiology as well as the pathophysiology of the dyslipidemias will emerge. The coordinate control of cholesterol synthesis through HMG-CoA reductase, cholesteryl ester hydrolysis by both acid and neutral cholesteryl hydrolyses, and the number and affinity of the different lipoprotein receptors in cellular membranes are of central importance in our research program. Using these techniques, the lipids, lipoproteins and their cellular metabolism have been evaluated in a number of disease states: familial hypercholesterolemia, abetalipoproteinemia, Wolman disease, cholesteryl ester storage disease and Tangier disease. We have determined that adult human liver expresses distinct recognition sites for apolipoproteins A-I, E, and B. By analyzing the hepatic receptors for these apolipoproteins in several dyslipidemic states, distinct genetic and physiologic control of these receptors has been demonstrated. Cellular abnormalities in cholesteryl ester hydrolysis and its relation with dyslipoproteinemia has also been explored. In addition to examining perturbations in cellular recognition and metabolism of lipoproteins, potential abnormalities in the biologic function of specific lipoproteins and apolipoproteins have been evaluated. In addition, clinical studies in the treatment of a variety of inborn errors in human lipid and lipoproteins are being conducted. The medications neomycin, niacin, cholestyramine and mevinolin are currently being studied in prospective, randomized, placebo-controlled trials. The use of bone marrow transplantation has been evaluated in Acid cholesteryl ester hydrolase deficiency.

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Project Description:

Objective:

1) To evaluate the interaction and metabolism of lipids and lipoproteins in liver from normal and dyslipidemic humans.

The liver plays a central role in mammalian lipid and lipoprotein metabolism. The liver is not only the site of cholesterol synthesis, it is also the primary cholesterol excretion site. Furthermore, the liver synthesizes and secretes apolipoproteins and intact lipoproteins and it also removes specific lipoproteins from plasma. Although study of hepatic lipid and lipoprotein metabolism in non-human mammalian species has led to insight into physiologic and biochemical mechanisms, the obvious interspecies variation in hepatic lipid and lipoprotein metabolism has limited an understanding of the role of the human liver in normal and aberrant lipoprotein metabolism. The first step in cellular-lipoprotein interaction is the binding of the lipoprotein to specific membrane receptors. As we reported last year, normal human liver specifically binds low density lipoproteins (LDL) while individuals lacking the LDL receptors in fibroblasts (familial hypercholesterolemic homozygotes) have altered hepatic LDL recognition. By extending these hepatic lipoprotein receptor studies to include the study of other lipoproteins, to study the lipoprotein binding in other dyslipidemic conditions, and by studying human hepatocyte lipoprotein receptor regulation, we have expanded our understanding of the role of lipoprotein receptors in human liver.

Methods Employed:

1) Using the hepatic membrane isolation techniques developed last year, hepatic membranes were prepared from hepatic tissue samples taken from normolipidemic subjects and subjects with homozygous familial hypercholesterolemia after portacaval anastomosis and abetalipoproteinemia.

2) Lipoproteins isolated by preparative ultracentrifugation and Geon-Pevikon Block electrophoresis were iodinated by the iodine monochloride method.

3) The assessment of the binding of iodinated and apolipoproteins and lipoproteins to the hepatic membrane preparations was performed utilizing a Beckman Airfuge. Hepatic apolipoprotein synthesis using ^{35}S-methionine containing media in hepatic organ culture was assessed utilizing autoradiography and immunoprecipitation.

4) Methods for culturing human hepatocytes *in vitro* were developed. Hepatocyte lipoprotein metabolism was studied in normal and homozygous familial hypercholesterolemic homozygotes adapting fibroblast lipoprotein receptor assay techniques.

Major Findings:

1) The apolipoprotein recognition of human liver is not the same as that observed by *in vitro* fibroblast assay techniques. Not only are protease sensitivity, calcium sensitivity, and K_D dissimilar, but also the total loss of the fibroblast LDL receptor in homozygous FH is paralleled by only a 50% decline

in LDL recognition by FH liver. Therefore, in vivo human liver has a distinctly different lipoprotein receptor system from that observed in peripheral cells.

2) The residual LDL binding observed in liver from FH homozygotes can be regulated. By performing a portacaval anastomosis, the hepatic recognition of LDL increased markedly and these changes paralleled alterations observed in the plasma LDL concentration as well as in the hepatic cholesterol and cholesteryl ester content.

3) In abetalipoproteinemia, an inborn error of metabolism in which no VLDL or LDL are present in the circulation, the hepatic recognition of LDL is markedly enhanced. This suggests that the hepatic LDL receptor(s) can be upregulated by both portacaval anastomosis as well as by depleting the pool of circulating apolipoprotein B.

4) By comparing the recognition of lipoproteins containing apoB, apoA-I, and apoE by hepatic membranes from normolipidemic subjects, FH homozygotes pre- and post-portacaval shunt, and in abetalipoproteinemia, an entire system of apolipoprotein recognition sites has emerged. ApoB binding is genetically distinct from apoE and apoA-I recognition. ApoE binding is enhanced and apoA-I binding depressed after portacaval shunt in FH. These observations indicate that human apolipoprotein recognition is a complex system of multiple lipoprotein recognition systems under separate physiologic and genetic control.

5) Modulation of human hepatocyte cholesterol content leads to alteration of both LDL and HDL receptors. The receptor number is coordinately regulated with HMG-CoA reductase activity. The in vitro hepatic membrane studies in normal and FH homozygotes were paralleled by studies performed in cultured hepatocytes from normal and FH patients. Similar to the attenuated LDL binding observed in the FH hepatic membranes, FH hepatocytes have diminished but not absent ability to bind, internalize, and degrade LDL. This nonsaturable, unregulated binding site parallels the metabolism of chemically modified LDL by normal human hepatocytes. Therefore, human liver manifests both a regulated, high affinity LDL receptor as well as a nonsaturable, unregulated LDL uptake mechanism which is independent of bulk phase pinocytosis.

6) It had been postulated that lipoproteins synthesized by the intestine during feeding are directed to the liver by a specific form of apoB, apoB-48, whereas the liver secretes lipoproteins with a different isoform of apoB-100, which is taken up by peripheral cells. Using human hepatic organ culture incubated in media containing ^{35}S-methionine, we determined that human liver indeed secretes apoB-100. However, apoB-48 was not produced. These findings are consistent with the separate biosynthesis of B-48 and B-100 by the intestine and liver, respectively.

7) In vivo metabolic studies indicated that apoA-$I_{Tangier}$ was cleared from the plasma more rapidly than normal apoA-I. All three systems demonstrated an increased binding and degradation of apoA-$I_{Tangier}$. These findings suggest for the depressed levels of HDL in Tangier disease are due to enhanced degradation of lipoproteins containing apoA-$I_{Tangier}$.

Objective:

2) To study the lipoproteins and lipoprotein receptors in an animal model for homozygous familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit.

By breeding a mutant rabbit with spontaneous hyperlipidemia and accelerated atherosclerosis, Dr. Y. Watanabe of Japan developed an animal model (WHHL) for spontaneous atherosclerosis. Preliminary studies by other workers indicated that the WHHL rabbits were good models for the LDL receptor-negative form of homozygous familial hypercholesterolemia. By evaluating clinical features, adrenal function, hepatic and fibroblast receptor studies, and characterization of the plasma apolipoproteins, the role of specific apolipoproteins or the clinical features observed in this animal model could be discerned.

Methods Employed:

In vitro fibroblast and hepatic membrane LDL receptor studies utilizing ^{125}I-LDL were conducted utilizing standard techniques. Adrenal reserve was assessed by measurement of serum corticosterone and cortisol before and after cosyntropin injection. Plasma lipids were quantified by standard techniques, and plasma apolipoproteins were evaluated by two-dimensional gel electrophoresis.

Adrenal and corneal histopathology and lipid content were assessed using standard techniques.

Major Findings:

1) Although the WHHL rabbit does spontaneously develop profound hyperlipidemia and accelerated atherosclerosis, the hepatic membrane and fibroblast studies are not indicative of a total loss of high affinity LDL receptors. Instead, a receptor which is defective rather than absent appears to be present.

2) The lipid and apolipoprotein evaluation disclosed patterns consistent with retention of chylomicron remnant particles in the circulation.

3) Steroidogenic tissues express LDL receptors in order to obtain LDL-derived cholesterol necessary for hormone synthesis. In this receptor-defective mutant cell line, steroidogenesis could be impaired due to the inability of the tissue to elaborate sufficient LDL receptors. Although fasting baseline cortisol and corticosterone concentrations in WHHL rabbits were the same as in normal rabbits, an attenuated response to ACTH stimulation was observed. These biochemical studies were paralleled by morphologic differences in adrenal tissue observed by light and electronmicroscopic studies. Therefore, under pharmacologic stimulation, this LDL receptor-defective mutant has impaired steroidogenic potential.

4) There has been no animal model in which to study the finding of arcus corneae which is observed in hyperlipidemic man. Using sensitive histopathologic as well as biochemical assays, cholesterol, cholesteryl ester, and triglyceride deposition at the corneal limbus was discerned in WHHL but not normolipidemic rabbits. Thus, a model for arcus corneae as well as a potential model for studying lipid and lipoprotein flux and egress has been defined.

Objective:

3) Evaluation of inborn errors in cholesteryl ester metabolism.

By evaluating tissues derived from individuals lacking a specific enzyme, the normal role of the enzyme as well as its relationship to other cellular processes can be deduced. A deficiency in acid ester hydrolase, a lysosomal enzyme, which hydrolyzes cholesteryl ester and triacylglycerol bonds, can lead to Wolman Disease and Cholesteryl Ester Storage Disease (CESD). Correlation of these in vitro studies with the different clinical presentations of these two diseases as well as a characterization of their circulating lipids and lipoproteins could provide an understanding of the role of lysosomal cholesteryl ester metabolism on lipoprotein physiology.

Methods Employed:

Assays for acid neutral cholesteryl ester hydrolase were developed and initially published last year. Cellular cholesterol, cholesteryl ester, and triacylglycerol content was determined using the sensitive enzyme, fluorimetric assay previously reported. Plasma lipids, lipoproteins, and apolipoprotein concentrations were quantitated using ultracentrifugation, colorimetric assays, and specific immunoassays.

Major Findings:

1) As reported last year, despite the loss of the acid esterase in Wolman Disease, the neutral esterase activity remained intact. We have subsequently found that in CESD the neutral esterase activity is increased. Therefore, neutral esterase is not only genetically distinct from acid esterase but the enhanced activity in CESD may play a role in the more mild clinical course experienced by these patients.

2) Acid esterase deficiency has a broad clinical presentation which does not appear to result from different underlying molecular mechanisms. By performing co-culture and cell fusion experiments utilizing fibroblasts derived from patients with CESD and Wolman Disease, no cross-correction of enzymic activity was found. Therefore, unlike a variety of other lysosomal storage diseases, the clinical spectrum observed in acid esterase deficiency cannot be ascribed to series of defects in enzyme metabolism which can be detected by co-culture or cell fusion techniques.

3) Marked changes in the plasma lipoproteins are observed in Acid Esterase Deficiency. These patients manifest type II hyperlipoproteinemia with increased concentrations of total cholesterol and LDL cholesterol which are in the upper 10th percentile for age and sex. This is paralleled by an increased apoB concentration and markedly decreased HDL concentrations. These alterations in plasma lipids and lipoproteins highlight the importance of lysosomal cholesterol delivery to the cell for normal lipoprotein metabolism.

4) The marked hepatomegaly and lipid accumulation observed in both CESD and Wolman Disease can now be sequentially evaluated noninvasively. The reduced radiodensity observed in the liver of these patients reflects the degree of lipid

accumulation in that organ. Therefore, a noninvasive tool to assess the impact of future therapy on hepatic lipid has been established.

5) The adrenal reserve in Wolman Disease is reduced because steroidogenic tissues utilizing LDL cholesterol for the sterol backbone in hormone synthesis, a defect in the ability of LDL cholesteryl esters to undergo hydrolysis, could result in impaired steroid synthesis. Although basal, fasting, serum cortisol concentrations were normal, a 36-hour ACTH infusion disclosed an attenuated rise in serum cortisol concentrations. These findings not only illustrate the importance of lysosomally derived cholesterol for normal adrenal function, they also indicate that during prolonged stress these patients may require steroid therapy.

6) The diagnosis of acid cholesteryl ester hydrolase deficiency in both Wolman Disease and CESD phenotypes can be made by evaluation of urine. Urinary renal tubular epithelial cells are shed in the urine of these subjects. Both compositional analysis and enzymic activity reliably provide the diagnosis in these diseases.

7) Repletion of Wolman and CESD fibroblasts with the enzyme acid lipase is possible. Since acid lipase is delivered to lysosomes by the mannose-6-phosphate receptor pathway, these studies suggest that enzyme replacement therapy or bone marrow transplantation could ameliorate the lipid accumulation in CESD and Wolman disease.

Objective:

4) Treatment of patients with inborn errors of lipid and lipoprotein metabolism.

Patients with a variety of inborn errors in lipid metabolism are followed in the outpatient clinic as well as on the inpatient service. We systematically evaluated the efficiency and/or mechanisms of action of treatment modalities in type II hyperlipoproteinemia, Wolman Disease, and homozygous familial hypercholesterolemia.

Methods Employed:

Standard plasma lipid and lipoprotein quantitation techniques were used. These included enzymatic, colorimetric assays combined with ultracentrifugation. Studies in the outpatient clinic included double-blind, randomized, placebo-controlled crossover clinical trials utilizing neomycin, neomycin and niacin, neomycin and cholestyramine, and mevinolin. Radiolabeled lipoprotein turnover studies on FH homozygote patients both before and after portacaval shunt were performed to evaluate the modulation of specific apolipoprotein receptor sites *in vivo*. Finally, bone marrow transplantation was attempted in Wolman Disease.

Major Findings:

1) Neomycin reduced total and LDL cholesterol concentration on an average of 20 and 24%, respectively, in patients with type II hyperlipoproteinemia without adversely affecting the protective HDL cholesterol concentration.

2) No ototoxicity, nephrotoxicity, or other serious side effects were detected with neomycin treatment.

3) Neomycin was as effective as other current treatment regimens in lowering LDL cholesterol concentration and has the advantages of little or no side effects, simply twice a day regimen, and a cost one-fourth to one-third that of conventional treatment.

4) Portacaval shunt reduced LDL cholesterol concentrations 15-25% in FH homozygotes and 36% in heterozygous FH.

5) By a sensitive agarose gel electrophoresis technique, heterogeneity in type II hyperlipoproteinemic study subjects was observed. This heterogeneity may reflect different underlying molecular defects, all of which result in a type II phenotype.

6) Although neomycin treatment altered the antibiotic resistance pattern of aerobic colonic flora, no broad-based antibiotic resistance which could represent a public health hazzard was detected.

7) Neomycin in combination with niacin normalized the plasma lipoprotein levels in 92% of type II hyperlipoproteinemic patients.

8) Portacaval shunt in FH homozygotes does not affect the normal $apoB_{48}$ and apoE metabolism in these patients with no functional LDL receptor. This suggests that intestinal chylomicron remnants are metabolized independent of the LDL receptor pathway.

9) Bone marrow transplantation has benefited some types of lysosomal storage disease. We attempted the first bone marrow transplantation in Wolman Disease in collaboration with the Department of Pediatrics at the University of Minnesota. The child died prior to full engraftment. Therefore, the first attempt to definitively treat Wolman Disease was unsuccessful. However, bone marrow transplantation remains a therapeutic possibility in this uniformly fatal disease.

Significance to Biomedical Research and the Program of the Institute:

By evaluating the interaction of lipoproteins with intact human cell lines and isolated subcellular fractions from different tissues in vitro, insights on the normal physiologic lipid transport function of the lipoproteins and their coordination with intracellular lipid metabolism can be derived. In addition, these studies can be extended to evaluate possible pathophysiologic mechanisms of the dyslipoproteinemias. Information on specific molecular defects in the dyslipidemias and the metabolic consequences of these defects is necessary for an understanding of these disease processes and may ultimately lead to more effective treatment for these disorders. In addition, these findings provide insight that could be more generalized to the understanding and prevention of atherosclerosis and coronary artery disease.

Proposed Course:

The studies on specific human cellular apolipoprotein receptors will be extended to include their isolation and characterization. In addition, utilizing cloned genes for the apolipoproteins, potential coordinate control of the cellular receptors and apolipoprotein transcription and translation will be investigated. The screening of patients with unique abnormalities in apolipoprotein structure will commence utilizing two-dimensional gel electrophoresis in the clinical lab. The studies related to mevinolin and neomycin therapy in type II hyperlipoproteinemia will continue.

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2. Meng, M.S., Gregg, R.E., Schaefer, E.J., Hoeg, J.M., and Brewer, H.B., Jr.: Presence of two forms of apolipoprotein B in patients with dyslipoproteinemia. J. Lipid Res. 24: 803-809, 1983.

3. Sprecher, D.L., Schaefer, E.J., Kent, K., Gregg, R.E., Zech, L.A., Hoeg, J.M., McManus, B., Roberts, W.C., and Brewer, H.B., Jr.: The cardiovascular features of homozygous familial hypercholesterolemia. Am. J. Cardiol. (in press).

4. Hill, S.C., Hoeg, J.M., Dwyer, A.J., Doppman, J.L., and Vucich, J.J.: CT findings in acid lipase deficiency: Wolman's disease and cholesteryl ester storage disease. J. Comput. Assist. Tomogr. 7: 815-818, 1983.

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6. Hoeg, J.M., Demosky, S.J., Jr., Schaefer, E.J., Starzl, T.E., and Brewer, H.B., Jr.: Characterization of hepatic low density lipoprotein binding and cholesterol metabolism in normal and familial hypercholesterolemic subjects. J. Clin. Invest. 73: 429-436, 1984.

7. Bieri, J.G., Hoeg, J.M., Schaefer, E.J., Zech, L.A., and Brewer, H.B., Jr.: The effect of vitamin replacement on plasma and hepatic vitamin A and E concentrations in abetalipoproteinemia. Ann. Intern. Med. 100: 238-239, 1984.

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9. Hoeg, J.M., Loriaux, L., Gregg, R.E., Green, W.R., and Brewer, H.B., Jr.: Impaired adrenal reserve in the Watanabe heritable hyperlipidemic rabbit: Implications for LDL receptor function in steroidogenesis. Metabolism (in press).

10. Hoeg, J.M., and Brewer, H.B., Jr.: Cutaneous manifestations of the dyslipoproteinemias. J. Assoc. Milit. Dermatol. (in press).

11. Hoeg, J.M., Demosky, S.J., Pescovitz, O.H., and Brewer, H.B., Jr.: Cholesteryl ester storage disease and Wolman Disease: phenotypic variants of lysosomal and cholesteryl ester hydrolase deficiency. Am. J. Human Genet. (in press).

12. Hoeg, J.M., Schaefer, E.J., Romano, C.A., Bou, E., Pikus, A.M., Zech, L.A., Bailey, K.R., Gregg, R.E., Wilson, P.W.F., Sprecher, D.L., Grimes, A.M., Sebring, N.G., Ayres, E.J., Jahn, C.E., and Brewer, H.B., Jr.: Neomycin favorably affects plasma lipoprotein concentrations in type II hyperlipoproteinemia. Clin. Pharm. Ther. (in press).

13. Tandon, N.N., Hoeg, J.M., and Jamieson, G.A.: Perfusion studies on the formation of mural thrombi with cholesterol-modified and hypercholesterolemic platelets. J. Lab. Clin. Med. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02024-03 MDB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Plasma Apolipoproteins and Lipoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Simon W. Law	Expert	MDB, NHLBI
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Richard Gregg	Medical Staff Fellow	MDB, NHLBI
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Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.3	5.3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A Tangier liver cDNA library was established in E. coli and two apoA-I cDNA clones were identified. Nucleic acid sequence analysis of these clones shown amino acid sequence 116-243 encoded by Tangier mRNA is identical to normal except at position 120 where there is one base change (G-T) thus changing glutamic acid at 120 to aspartic acid. This base substitution also create a new Sau3Al restriction site. The importance of this amino acid substitution is currently underway.

In collaboration with Drs. A. Sakaguchi and S. Naylor, we have used the cloned apoA-I cDNA as hybridization probe in Southern blot assay of human-mouse somatic cell hybrid DNA to localize the apoA-I and apoC-III gene to the p11-q13 region of human chromosome 11. The study represents the first definitive assignment of an apolipoprotein gene to human chromosome. In a seperate Southern blot analysis, we identified genetic differences in kindreds with apoA-I and apoC-III deficiency.

Two additional apolipoproteins, A-II and C-II have been cloned. Nucleic acid sequence analysis showed these apoproteins are initially synthesized as precursor proteins. Apolipoprotein A-II is initially synthesized as preproapo A-II with a 23 amino acid preprosequence. PreproapoA-II then undergoes co-translational and post-translational cleavage to mature 77 amino acid long apoA-II. Apolipoprotein C-II is initially synthesized as preapoC-II with a 22 amino acid presequence. PreapoC-II then undergoes co-translational cleavage to mature 79 amino acid long apoC-II.

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Project Description:

Objectives:

1) Chromosomal Localization of Human Apolipoprotein A-I and C-III.

Methods Employed:

Chromosomal localization of the apoA-I gene has been determined by filter hybridization analysis of human-mouse hybrid cell DNAs containing known human chromosome content using nick-translated apoA-I cDNA as probe. Human-mouse somatic cell hybrids were contructed by fusing LM/K (thymidine kinase deficient) or RAG (hypoxanthine phosphoribosyl transferase deficient) fibroblasts with human fibroblasts or leukocytes. Proliferating cell hybrids were selected in hypoxanthine-aminopterin-thymidine medium (HAT). Cell hybrids were characterized for human chromosome content by trypsin-Giemsa banding. High molecular DNAs were isolated from these cell hybrids by SDS/proteinanse K treatment. Restriction endonuclease fragments were separated by 0.7% agarose gel electrophoresis at 35 volts for 16 hours, transferred to nitrocellulose filters and hybridized to apoA-I cDNA probe as described by Southern.

Major Findings:

The gene for apoA-I has been localized to the p11-q13 region of chromosome 11 by filter hybridization analysis of human-mouse hybrid cell DNAs containing chromosome 11 translocations utilizing a cloned human apoA-I cDNA probe. The known linkage of apoA-I and apoC-III also permitted the simultaneous assignment of the apoC-III gene to the same region on chromosome 11. Comparison with previously established gene linkages on mouse and human genome suggest that apoA-I and apoC-III may be linked to the esterase A4 and uroporphyrinogen synthase genes which are present on the long arm of human chromosome 11. The localization of the apoA-I and apoC-III genes in the p11-q13 region of chromosome 11 represents the first definitive chromosomal assignment of a human apolipoprotein gene will now enable more detailed analysis of the genome organization and linkages of the apolipoprotein genes.

Objective:

2) Cloning of Tangier apoA-I cDNA

Methods Employed:

Messenger RNA (mRNA) was isolated from liver biopsy using the quanidine thiocyante/Cscl cushion centrifugation procedure. A liver cDNA library was established in E. coli strain RRI and HB101 using the plasmid pBR322 as cloning vector. ApoA-I specific DNA fragment was isolated from a clone we have previously described and used as hybridization probe to screen for the Tangier apoA-I cDNA clones. Plasmid DNA were isolated by the Cscl density gradient procedure and DNA sequences were determined by the Maxam-Gilbert procedure.

Major Findings:

The normal apoA-I probe hybridized to two Tangier cDNA clones. One clone, pMDB136T was found to contain an insert DNA of 500 bp. DNA sequence analysis showed this clone included apoA-I amino acids 116 to 243. Only one base difference was found between normal and Tangier apoA-I mRNA at amino acid 120 (G-T) thus changing a glutamic acid residue (GAG) to an aspartic acid residue (GAT). The other clone, pMDB3628T was found to contain an insert DNA of 200 bp. DNA sequence analysis showed this clone included apoA-I amino acids 181 to 232 which correspond exactly to the normal apoA-I sequence.

Objective:

3) Cloning of apoA-II cDNA

Methods Employed:

Synthetic oligodeoxynucleotides were used as hybridization probes to select for apoA-II cDNA clones in a normal liver cDNA library prepared by this laboratory. Three sets of 14 bases long oligodeoxynucleotides containing apoA-II specific sequences were employed. ApoA-II cDNA clones were isolated by CsCl density gradient and nucleic sequence was determined by the Maxam-Gilbert procedure.

Major Findings:

Four apoA-II cDNA clones were isolated by Dr. Karl Lackner of this laboratory. Our result showed apoA-II is synthesized like apoA-I, as a precursor protein, preproapoA-II. PreproapoA-II contains a 100 amino acid propeptide. Unlike proapoA-I the cleavage site of proapoA-II is similar to other propeptides which are cleaved at a tryptic-like cleavage site. Thus, apoA-II precursor undergoes intracellular co-translational and post-translational cleavage to mature apoA-II.

Objective:

4) Cloning of apoC-II cDNA

Methods Employed:

A set of 17 bases long apoC-II specific synthetic oligodeoxynuclotides were employed as hybridization probes to select for apoC-II cDNA clones. ApoC-II cDNA clones were isolated by CsCl density gradient and nucleic acid sequence determiend by the Maxam-Gilbert procedure.

Major Findings:

ApoC-II cDNA clone was isolated by Dr. Silvia Fojo of this laboratory and the complete nucleic acid sequence determined. Our result showed apoC-II is synthesized as 101 amino acid preapoC-II. PreapoC-II contains a 22 amino acid prepeptide and no propeptide. Southern blot analysis of chromosomal DNA isolated from normal volunteer and patients with apoC-II absence showed identical restriction fragments.

Significance to Biomedical Research and the Program of the Institute

The cloning and nucleic acid sequence analysis of human apolipoproteins already provided new insights to the biosynthesis and processing of the plasma apolipoproteins. Using cloned cDNA as hybridization probes in Southern blot analysis enabled us to 1) identify genetic differences in two kindreds with apoA-I and apoC-III deficiency 2) assign apoA-I and apoC-III genes to specific region of chromosome 11. The use of molecular biology techniques will be most useful in establishing normal structure, function, regulation and linkages of apolipoprotein genes and in elucidating the molecular defects in patients with dyslipoproteinemias.

Proposed Course:

Studies will be continued on the identification of various apoA-I and apoC-II structural gene defects. We will use the various apolipoprotein cDNA clones as hybridization probes to 1) isolate genomic DNA clones from bacterial phage libraries to obtain a more detail knowledge of the structural organization of these apolipoprotein genes 2) to map chromosome location of the apolipoprotein genes 3) to analysis restriction polymorphism and linkage analysis.

Identification of other apolipoprotein cDNA are also underway. Their biosynthesis and processing will be monitored by both structural analysis and by in vitro translation system.

Publications:

1. Brewer, H.B., Jr., Fairwell, T, Kay, L., Meng, M., Ronan, R., Law, S.W., and Light, J.A. Human plasma proapoA-I isolation and amino terminal sequence. Biochem. Biophys. Res. Commun. 113, 626-632, (1983).

2. Law, S.W., and Brewer, H.B., Jr. Nucleotide sequence and the encoded amino acids of human apolipoprotein A-I mRNA. Proc. Natl. Acad. Sci. USA 81, 66-70, (1984).

3. Law, S.W., Gray, G., and Brewer, H.B., Jr. cDNA cloning of human apoA-I mRNA. Biochem. Biophys. Res. Commun. 112, 257-264, (1983).

4. Law, S.W., Gray, G., and Brewer, H.B., Jr. Sakaguchi, A., and Naylor, S. Human apolipoprotein A-I and C-III genes resides in the p11→q13 region of chromosome 11. Biochem. Biophys. Res. Commun. 118, 934-942, (1984).

5. Minghetti, P., Law, S.W., and Dugaiczyk, A., In the alphafeto-protein-albumin family. The molecular evalutingary clock runs faster for alphafetprotein. J. Molecular Bio. and Evolution (in press).

6. Fojo, S.S., Law, S.W., Brewer, H.B. Jr. Human apolipoprotein C-II complete nucleic acid sequence of preapoC-II. Proc. Natl. Acad. Sci. USA (in press).

7. Lackner, K.J., Law, S.W., and Brewer, H.B. Jr. Human apolipoprotein A-II: Complete nucleic acid sequence of preproapoA-II. FEBS Letters (in press).

8. Fojo, S., Law, S.W., Brewer, H.B. Jr., Sakaguchi, A.Y., Naylor, S.L.: The localization of the gene for apolipoprotein C-II to chromosome 19. Biochem. Biophys. Res. Commun. (in press).

9. Lackner, K.TJ., Law, S.W., Brewer, H.B. Jr., Sakaguchi, A.Y., Naylor, S.L.: The human apolipoprotein A-II gene is located on chromosome 1. Biochem. Biophys. Res. Commun. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02026-01 MDB

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Coronary Physiology, Hyperlipoproteinemia, and Atherosclerosis in a Miniswine Model

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI: Dennis L. Sprecher	Medical Staff Fellow	MDB, NHLBI
Others:		
H. Bryan Brewer Jr	Chief	MDB, NHLBI
Stephen Epstein	Chief	CB, NHLBI
Randolph Patterson	Senior Investigator	CB, NHLBI
Joseph Pierce	Chief	
Howard Kruth	Chief	EA, NHLBI

COOPERATING UNITS *(if any)*

Peter Ramwell, Dept. of Physiology, University of Georgetown

LAB/BRANCH

Molecular Disease Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.6	1.6	1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Preliminary evidence suggests that there is a difference in the vasoactive response in the coronary arteries of miniswine to various pharmacologic agents. Significant changes can be observed with thromboxane like drugs, histamine, ergonovine maleate, and acetylcholine. Hyperlipidemia and/or atherosclerosis in coronary vessels of miniswine alter the normal vasodilating response to histamine. Acetylcholine may have a potentiated vasoconstricting effect on atherosclerotic vessels but not necessarily in all hyperlipidemic animals. Finally, the response of the coronary vessel to various receptor mediated agonists is highly heterogeneous between animals, even those that are genetically similar.

In-vitro coronary vessel preparations do not necessarily correlate with results derived from in-vivo preparations. Histamine and acetylcholine responses are diametrically opposed to those seen in the intact animal. Furthermore, in-vitro contractile responses to histamine are different in the right coronary artery, vs the two left coronary vessels. Isolated muscular preparations of coronary vessels also appear to be affected by low density lipoprotein in the bath solution.

A plasma two-dimensonal gel system has been completed to elucidate apolipoprotein changes in miniswine with feeding, as well as genetic defects in the human apolipoproteins. These alterations in apolipoproteins with high fat feeding in miniswine correlate with the resulting hyper and hyporesponder defined by plasma cholesterol concentrations.

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Project Description

Objective: The current research of this laboratory is the following:

1) a) To determine whether atherosclerotic vessels of miniswine produce a greater alteration in coronary vascular resistance to various injected drugs than normal control coronary arteries.
   b) To determine the correlation between the lipid values and the coronary flow values obtained with drug injection.
   c) To determine the apolipoprotein changes observed with high cholesterol, high fat feeding in miniswine; and to correlate these changes to the lipoprotein values, vascular pathology, and drug induced flow alterations.

Methods Employed:

All male miniswine (Hormell), ages 1-2 years, between 60 and 130 kgs are placed on a specific diet and brought to surgery after a specified amount of time. After induction of anesthesia with ketamine, intubation is performed and a maintenance halothane/nitrous oxide anesthesia is given. A left lateral thoracotomy, in the forth intercostal space is accomplished with electrical cautery, and the pericardial sac is exposed. Careful incision of the pericardium exposes just the right atrial appendage and the left anterior descending coronary artery. Dissection of the LAD just distal to the takeoff of the circumflex is done, and a electromagnetic flow probe and distal balloon occluder placed. A 27 guage lymphangiogram needle is then inserted approximately 1-2 cm distal to the flow probe, in a retrograde fashion, and continuous flush provided to prevent clotting at the needle tip. Drugs are injected through the lymphangiogram needle in 2 cc aliquots over a 45 second period. Flow measurements are recorded on a brush recorder, as well as mean and phasic aortic pressure (from catheter in left carotid). The left internal jugular vein is also cannulated for venous access and continuous normal saline drip. EKG recording provides heart rate and indication of ST segment alterations. Termination of experiment includes accurate measurements of flow probe and injection site locations on coronary vessel and removal of heart for future pathology.

Lipoprotein concentrations are evaluated using the standard LRC methodology. Only cholesterol, triglycerides, and HDL cholesterol values are being determined. A reasonable correlation has been observed between the precipitation technique for HDL cholesterol determinations and the concentrations determined after ultracentrifugation.

Apolipoprotein concentrations are determined by computer image processing of two-dimensional polyacrylamide gels. Vascular pathology is analyzed by utilizing both lipid staining and microscopic techniques.

Major Findings:

35 pigs were divided into the following categories: 14 animals for controls, 7 animals for one month high fat, high cholesterol diets, and 14 animals for over 6 months of high fat, high cholesterol diets: i.e. controls,

hyperlipidemics and hyperlipidemics/atherosclerotics. Animals undergo surgery once a week, where they are terminated. Drugs used for injection are U46619 (a thromboxane like drug), histamine, ergonovine maleate, methoxamine, acetyl choline, and arginine-8-vasopressin, in that order. These drugs thus test respectively thromboxane receptors, histamine receptors H1 and H2, alpha and/or serotenergic receptors, alpha 1 receptors, cholinergic receptors, and possibly other prostaglandin mediated receptors. They are injected with 5 minute intervals at least, or longer if the coronary flow did not return to baseline. Coronary vascular resistance (CVR) was calculated from flow measurement and aortic pressure (means) for each drug. Either percentage vasodilation or vasoconstriction was calculated. Mean values for each experimental group are then obtained. Preliminary results of the control vs the hyperlipidemic group indicate that acetylcholine produces more of a flow reduction in animals with hyperlipidemia. It appears that those animals who respond most dramatically to diet with hypercholesterolemia appear to have the most marked reduction in flow with acetylcholine. Pathologic evaluation indicates great heterogeneity in the amount of disease. In addition, all animals with sudan staining lesions macroscopically had potentiated responses to acetylcholine. No animals with absence of microscopic disease had an elevated acetylcholine response. In addition, it can be concluded that simply elevated plasma cholesterol levels do not predispose to enhanced responsivity to the drugs injected. Final timed controls and the atherosclerotic group have not been studied yet and thus final results must await their outcome.

The apolipoprotein concentrations oscillate as do the cholesterol levels over the initial months the animals are fed a high fat, high cholesterol diet. However, within the first 4 months, the animals clearly divide into a group which sustains a marked elevation in HDL cholesterol and apoA-I, (the hyperresponder), while others do not sustain a high HDL cholesterol, suggesting the hyporesponder (i.e., not synthesizing the HDL particle). ApoA-IV concentrations correlate with high fat feeding, but do not necessarily correlate with plasma triglyceride levels. Quantification of these results await final data and computer analysis.

Objective:

2) To determine whether various preparations of activated platelets alter flow in the coronary vessels.

Methods Employed:

Platelets are gathered the morning of pig surgery, after only Ketamine anesthesia, and processed over the ensuing 2-3 hours while the pig is being prepared with appropriate hardware for coronary flow studies. Dr. Joseph Fratantoni makes platelet rich plasma from blood collected in citrate, and produces aliquots for intracoronary injection.

Major Findings:

Conclusions are pending further animal studies.

Objective:

3) To determine in vitro vascular response to drugs between right and left coronary vessels in miniswine, and alteration in response when endothelium is removed from the vessel. In addition, elucidating the alteration in response to pharmacologic agents when the vessels are made atherosclerotic.

Methods Employed:

Miniswine are prepared as in study #1, however, after anesthesia, the heart is rapidly exposed, major vessels clamped, and the heart removed. It is then washed in iced lactated ringers solution and placed in a iced bucket surrounded by ringers solution. This is then transferred to a physiology laboratory within 30 minutes where the coronary vessels are rapidly dissected for in vitro studies. 3 mm ring segments are then taken at specified distances from the ostia and strung up on strain guages in baths of physiologic solution. Pharmacologic evaluation is performed by adding increasing doses in the bath, and watching the change in tension across the tissue. Results provide quantification of sensitivity to the drug and maximal tension developed. Endothelial removal is done by careful rubbing of intima with blunt forceps. Transmission electron microscopy and silver staining indicate absence of endothelium with this technique.

Major Findings

Four carefully prepared hearts were evaluated. From each vessel, 7 mm from the ostia, two 3 mm rings were taken, one for an intact study and one for a deendothelialized study. Thus, a total of 6 rings from each heart, or 24 pieces from the four hearts. Three drugs in sequence were used: U46619, histamine, and acetylcholine. U46619 gave results similar to histamine, however, histamine produced more dramatic results. Acetylcholine, the response to which is known to indicate endothelial removal, contracted and then relaxed almost all of the vessels, suggesting prior drugs may have altered its known effects. Three major conclusions were made: a) the maximal tension produced by histamine in the right coronary was significantly less ($p < 0.025$) than that generated by either the left circumflex or left anterior descending arteries, b) intimal rubbing produced a significant increase ($p < 0.25$) in the maximum force generated in the right coronary artery (factor of 2.5), but not in the other two arteries, and c) sensitivities between vessels whether intact or rubbed were not significantly different, i.e., concentration of histamine to produce 50% of maximal tension.

Objective:

4) In vitro incubation studies, to elucidate the alteration in coronary contractile response when low density lipoprotein particles are present.

Methods Employed:

Preparation of tissue is as in #5 above. A vessel is then stimulated with a particular drug, whereupon after a one hour incubation period the vessel is again exposed to the same concentration of drug. Superb reproducibility was

achieved in normal physiologic solutions. Low density lipoprotein was prepared from normal volunteers after plasmapheresis. A 1.030 to 1.050 g/cc density fraction was used to maximize homogeneity of LDL. Dialysis was then performed with the physiologic bath solution used as dialysate.

Major Findings:

Only preliminary results are available. Intact vessels do not alter their response to histamine or acetylcholine after one hour of incubation in LDL solution. However, denuded pieces have an enhanced response to histamine after a one hour LDL incubation.

Objective:

5) In vivo modulation of specific coronary vasoconstrictor response to endoperoxide by ergonovine maleate and arginine-8-vasopressin in miniswine.

Methods Employed:

Methods used are as in #1 above.

Major Findings:

Using 10 miniswine, U46619 was used at three different dosages before and after ergonovine maleate and arginine-8-vasopressin. Several conclusions were drawn: 1) U46619 produced a dose response curve which fit the line 32.2 x (dose in micrograms) + 3.8 ($r = 0.79$, $p < 0.02$, $n = 15$); 2) ergonovine maleate (.2 mg) produced a 24 ± 13% increase in coronary vascular resistance (CVR); 3) arginine-8-vasopressin (0.05 micrograms) produced a 23 % 16% increase in CVR; 4) the dose response curve of U46619 after the two modulating drugs was enhanced ($p = 0.05$).

Objective:

6) Develop a two-dimensional plasma gel electrophoresis system to elucidate the apolipoproteins in both humans and miniswine.

Methods Employed:

The O'Farrel technique was modified to more easily observe the apolipoproteins usually evaluated in humans. A highly sensitive silver stain was utilized to assist in analyzing the various isoforms of these proteins. The gel have undergone image processing to record the intensity of spots. The computer program used to quantify spot intensity was written by Dr. Mark Miller, of NCI.

Major Finding:

Human apolipoproteins A-I, A-II, A-IV, C-II, C-III, D, E, and H can be seen on one plasma electrophoretogram (pI = 4.7). ApoC-I had a pI of 8.0, and thus was not seen on the original map. ApoB can only be adequately visualized on a

3% acrylamide gel due to its large molecular weight. The major desialated form of apoB has an apparent molecular weight of 30,000 daltons and has a pI of 5.3. ApoH has sialated forms at MW 48,000 daltons, pI ranging from 6.5 to 7.0. The major desialated from has a pI of 9.5

Objective:

7) Evaluate the clinical and biochemical history of homozygous familial hypercholesterolemic patients followed at the NIH for over 15 years.

Methods Employed:

Sixteen FH homozygotes followed at the NIH were analyzed per clinical history, lipoprotein profile, angiography, and residual LDL-receptor activity performed on fibroblasts in tissue culture. Pathologic evaluation was also performed on the 4 deceased patients.

Major Findings:

The nine asymptomatic patients had lower total cholesterol levels, higher residual receptor activities, and a greater percentage of females than the seven symptomatic patients. Coronary ostial disease and aortic root cholesteryl ester deposition appeared to be highly associated with premature cardiac symptoms. Femoral bruits were found as a typical antecedent to cardiac symptoms.

Significance to Biomedical Research and the Program of the Institute

Coronary artery disease is the leading cause of death in the United States. Functional loss due to this disorder is enormously high. It is clear that patients with significant angiography proven coronary arterial narrowing have varying amounts of associated discomfort and varying probabilities of developing a myocardial infarction. In addition, there are those patients without any documented coronary narrowing who have either angina or past myocardial infarction. The assumed block in coronary flow has been shown in some cases to be transient large vessel spasm. This dynamic component of the coronary vasculature may be the basis for cardiac symptoms or ultimate myocardial necrosis unassociated necessarily with the exact amount of fixed vascular narrowing. An understanding of this mechanism may lead to more successful treatment regimens for angina patients. The suggestion that atherosclerosis per se makes a vessel more responsive to particular agonists indicates either an alteration in lipid-membrane constitutents, change in endothelial secretagogues, or an alteration in endothelial-smooth muscle interaction. Since elevated lipid levels themselves without intimal thickening or noted sudan staining do not appear to enhance the vascular responsivity, it suggests that clear endothelial and/or smooth muscle alteration is essential. This may be related to the mechanism of a barrier to the passage between plasma, and smooth muscle, or curtailment of the manufacture of products from endothelial cells which serve to relax smooth muscle. The correlation between apolipoproteins, particularly apoA-I and the presence of coronary disease suggests the possible connection between decrease in apoA-I and elevation in vascular tone. Perhaps apoA-I is a marker for lipid egress from the cell, or from the cell membrane. If lipid over

time breaks down the endothelial lining than lipid lowering may markedly reduce the vascular responsivity to various plasma "hormones" which attach on to the endothelial receptors. The hemodynamic changes seen with progressive atherosclerosis presents a link between the hemodynamicist and the biochemist in elucidating the role of hyperlipidemia in coronary physiology.

Proposed Course:

The major study on miniswine will be completed and analyzed. This will be correlated with the in-vitro studies to elucidate the association between epicardial vessel dynamics and receptor response with hyperlipidemia, change in apolipoproteins, and pathologic vascular alteration especially in the intima. Once it is established that hyperlipidemia and/or atherosclerosis increases the response to any particular agonist, than particular receptor studies need to be undertaken. Endothelial cell culture, in the lipid laden and depleted state would be evaluated for alteration in receptor regulation or other synthesis products. Membrane analysis for phospholipid would gain insight into the alteration this constituent may have on receptor binding. Two-dimensional electrophoresis on either or both smooth muscle and endothelial cells would allow protein analysis and isolation in various hyperlipidemic states. Thus, the analysis of the constituent factors involved in presumably altered vascular tone could be analyzed and its mechanism perhaps effectively blocked. This would be a beginning towards an effective treatment for enhanced vascular tone and its associated clinical symptoms.

Publications:

1. Sprecher DL, Schaefer ES, Kent KM, Zech LA, Gregg RE, Hoeg JS, McManus B, Roberts WC, Brewer HB Jr. Cardiovascular features of homozygous familial hypercholesterolemia: Analysis of 16 patients. Am J. Cardiol 54:20-30, 1984.

2. Sprecher DL, Taam L, Brewer HB Jr. Two-dimensional analysis of human plasma apolipoproteins. Clin Chem. (December, 1984, in press).

Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

The Laboratory of Molecular Hematology (LMH) is involved in studying the basic molecular mechanisms of gene expression and protein synthesis, specifically using hemoglobin as a model system. LMH is composed of three segments: the Section on Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Section on Molecular Cloning, which is primarily concerned with the isolation and characterization of regulatory sequences that control globin and other genes from the genomes of eukaryotic cells; and the Section on Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the transcriptional and translational levels. LMH is closely associated with the Clinical Hematology Branch (CHB) and collaborates on a number of joint projects.

The objectives of this laboratory are to: (1) identify, isolate and characterize the regulatory factors of animal and human tissues that are involved in the control of the expression of the genes, particularly the globin genes; (2) analyze the genomic DNA sequences involved in the regulation of gene expression in humans and animals; (3) develop methods for transferring functional genes into tissue culture cells and intact animals; (4) develop and characterize animal and tissue culture model systems for human genetic anemias; (5) characterize the molecular basis of translational regulation; and (6) examine the overall regulation of gene expression in normal, experimental, and disease (e.g., thalassemia) states. Information from these programs is used to study the regulation of globin gene expression in normal and thalassemic mouse (and human) DNA. The long-term goal is to develop a means whereby globin gene expression can be corrected in mice (and, ultimately, in patients) with β-thalassemia and other diseases involving abnormal hemoglobin biosynthesis.

Factors involved in controlling the expression of the globin genes have been identified by a combination of cell biology and molecular biology techniques. Somatic cell hybrids, obtained by fusion of human or animal cells with mouse erythroleukemia (MEL) cells, have been used to provide evidence for positive and negative regulatory factors controlling globin gene expression. In order to purify and characterize these putative regulatory factors, intact-cell and cell-free assays have been established. A major regulator of gene expression in our system is methylation of DNA sequences flanking the globin genes. A number of studies are examining the methylation status and gene activity of exogenous genes in tissue culture cells and in intact animals. The methylase inhibitor 5-azacytidine is also being evaluated in these studies.

Several methods of gene transfer are being used to insert genes into tissue culture cells, bone marrow cells, and mouse embryos. The most successful of these has been the technique of physical microinjection in which one or more copies of a specific gene can be injected into the nucleus of a single cell under conditions whereby the injected cell (or fertilized egg) can be grown in culture (or in a surrogate mother) into a cloned population (or a living animal). This technique was successfully used a) to correct a mouse thymidine kinase (TK) negative L cell by microinjection of a bacterial plasmid containing a functional TK gene, and b) to insert plasmid DNA into the germ line of mice.

Retroviral techniques and recombinant DNA technology have been used to construct both retroviral vectors as well as "expression vectors" containing the globin gene regulatory regions. These vectors are plasmids designed to increase expression of the inserted gene. They contain various regulatory and control regions as well as a coding sequence.

The Laboratory has succeeded, during the past year, in demonstrating that:

(1) The promoter has a marked influence on the stimulatory effect of enhancing sequences as determined by using expression vectors containing regulatory and enhancing sequences.

(2) A number of different cloned genes can be efficiently inserted into mouse eggs or tissue culture cells by means of physical microinjection or retroviral vectors.

(3) The fragment of DNA containing the deletion of the β-major globin gene in the β-thalassemic mouse has, surprisingly, a 68 bp insertion that resembles a transposon-like element.

(4) Distinct RNA polymerase II transcription initiation and elongation complexes can be formed and isolated on the Adenovirus 2 major late promoter.

(5) At least two transcription initiation factors are stably bound to the preinitiation complex. One factor is released upon phosphodiester bond formation and can be reutilized for transcription of new DNA templates.

(6) A 60,000 dalton RNA polymerase II transcription initiation factor can be identified in functional transcription initiation complexes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02213-07 MH

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of RNA and Protein Biosynthesis in Cell-Free Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Safer	Medical Officer	LMH, NHLBI
Others:	W. F. Anderson	Chief	LMH, NHLBI
	H. E. Tolunay	Senior Staff Fellow	LMH, NHLBI
	T. Brendler	Staff Fellow	LMH, NHLBI
	M. Schafer	Chemist	LMH, NHLBI
	W. Kemper	Chemist	LMH, NHLBI
	L. Yang	Biologist	LMH, NHLBI
	M. J. Curcio	Biol. Lab. Tech.	LMH, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Metabolism, DIR, NHLBI, NIH (J. Moss)

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section on Protein Biosynthesis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.6	3.2	3.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Regulation of gene expression is controlled at the levels of transcription, processing, transport, and mRNA translation. The primary goals of this Section are to investigate the transcriptional and translational control mechanisms which mediate such controls. Procedures have been developed to divide the process of transcription initiation into distinct stages. This has allowed the extensive purification of stable, active, RNA polymerase II transcription initiation complexes by glycerol gradient centrifugation and gel filtration. Several transcription initiation factors are stably bound to the binary RNA polymerase II•DNA complex. A 60,000 dalton phosphoprotein specific for active transcription initiation at the Adenovirus 2 major late promoter cap site has been identified and is currently being purified and characterized.

To understand the function of eIF-2 subunits during ternary complex formation and guanine nucleotide exchange, and the regulation of these activities by phosphorylation/dephosphorylation mechanisms, the genes for the α, β and γ subunits of eIF-2 are being sequenced. HPLC procedures for the large scale isolation of eIF-2 subunits have been developed and the amino-terminal sequences of the α and γ subunits determined. Oligonucleotide probes based on these sequences are currently being used to screen cDNA and genomic libraries. The α and β subunits of eIF-2 have been shown to participate in guanine nucleotide exchange through their interaction with eIF-2B. The effect of ADP-ribosylation of eIF-2 and eIF-2B on this interaction is being evaluated.

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Project Description:

Objectives: The major goals are: (1) to determine the sites and mechanisms of transcriptional and translational control of gene expression in vitro; (2) to identify and characterize the components involved in such regulation; (3) to develop active in vitro translation and transcription systems which retain key regulatory features found in intact cells; and (4) to clone and sequence genes for eukaryotic translational initiation factors, initially eIF-2.

Methods Employed: Conventional chromatographic procedures, HPLC reverse phase and affinity chromatography using antibodies prepared against purified initiation factors are used to isolate rapidly key translational and transcriptional components under defined experimental conditions. Covalent modification of these regulatory factors will be examined by incorporation of specific radioisotopes, direct chemical analogs, group specific reagents, and by alteration of their physical characteristics (e.g., pI, S_{20w}). Active translation and transcription systems will be developed for K-562 cells using non-disruptive procedures which maintain cell ultrastructure. Large scale culture of K-562 cells will be used to obtain the large amounts of cells required for purification of translational and transcriptional components. Proteins are subjectd to automated Edman degradation to generate N-terminal amino acid sequence information. These partial sequences are used to synthesize mixed oligonucleotide probes using automated solid phase phosphotriester chemistry. The probes are purified via HPLC reverse phase chromatography. Urea-PAGE gels are used to check purity and probe size. The probes are tested for hybridization via Southern blot analysis. The eIF-2 probes will then be used to screen genomic and cDNA libraries. The selected clones will be evaluated via restriction enzyme mapping, hybridization-selected mRNA translation and partial sequence analysis. Once the desired genes have been isolated, sequence analysis and R-loop analysis can be utilized to elucidate the genetic organizational structure and the sequences responsible for the primary structure of the eIF-2 protein.

Major Findings:

1. Procedures have been developed for the formation and isolation of distinct RNA polymerase II transcription initiation and elongation complexes on Adenovirus 2 templates containing the major late promoter.

2. Assembly of transcription components required for correct initiation requires specific promoter sequences on the Ad2 MLP template.

3. Transcription initiation complex assembly requires ATP. A limiting component is sequestered by the complex in an ATP independent manner such that the subsequent addition of a second DNA template does not result in its transcription.

4. Elongation of preformed transcription initiation complexes releases these sequestered factors and allows their random association with available DNA templates during subsequent rounds of initiation.

5. A 60,000 dalton RNA polymerase II transcription initiation factor has been identified and is currently being purified and characterized.

Major Findings (continued):

6. The eIF-2 subunits, α, β and γ can be rapidly and simultaneously purified to a greater than 95% purity using reverse phase HPLC.

7. Quantitation of the purified subunits via amino acid composition analysis gave the expected 1:1:1 ratio and confirmed the molecular weights of α, β and γ to be 32, 35 and 55 Da, respectively.

8. The sequence of the first 23 amino acids and the first 7 amino acids from the N-terminal of the eIF-2α and the eIF-2γ subunits, respectively, was determined via automated Edman degradation. A preliminary attempt yielded no sequence information for the eIF-2β. This subunit may have a blocked NH_2 terminus.

9. Two mixed probes, a 17 mer composed of 24 chains and a 14 mer composed of 48 chains representing two distinct regions within the N-terminal of the eIF-2α subunit, were successfully synthesized. These were purified via reverse phase HPLC and evaluated via PAGE analysis.

Significance to Biomedical Research and the Program of the Institute:

Although regulation at the level of transcription is currently thought to be the primary mechanism for regulating the flow of genetic information, modulation of protein synthesis and interactions between translational and transcriptional components have recently assumed increased importance. Translational regulation has been identified as a major feature of host virus interaction. Regulation of protein synthesis by hemin was once thought to be a highly specialized means of coordinating globin chain and hemin synthesis in reticulocytes; it now appears, however, to be a widespread mechanism for post-translational modulation of gene expression involving a cascade of highly specific protein kinases and other covalent modifiers. Final control of gene expression during cell differentiation may also be regulated by interaction of translational components with messenger RNA, which, in turn, may interact with and be regulated by changes in the ultrastructure of the cell. It is essential, therefore, to understand the basic mechanisms involved in these processes to be able to control gene expression in the cell.

Proposed Course of the Project:

1. Polypeptides identified as components of RNA polymerase II initiation and elongation complexes will be purified from transcriptionally active nuclear extracts of HeLa and K-562 cells. Monoclonal antibodies prepared against transcription complex components will be tested for inhibition of transcriptional activity. The specific targets of these antibodies will then be purified by large scale affinity chromatography. The ultimate goal will be to assign specific functions to these transcriptional components required for the initiation process.

2. New translational and transcriptional systems are being developed to obtain *in vitro* systems which more closely approximate *in vivo* rates. Approaches being used include cell permeabilization techniques and new methods of cell disruption.

Proposed Course of the Project (continued):

3. Mixed oligonucleotide probes based on partial amino acid sequence data will be used to probe cDNA and genomic libraries for translation and transcription factors. The primary structure of these proteins, their genetic organization and control of their expression will be determined.

Publications:

1. Safer, B.: Regulation of eIF-2B mediated guanine nucleotide exchange by limited phosphorylation of eIF-2α. In Gene Expression: The Translational Step and Its Control (B.F.C. Clark and H. U. Pedersen, eds.) pp. 483-501, Munksgaard, Copenhagen, 1984.

2. Crouch, D. and Safer, B.: The association of eIF-2 with Met-tRNA$_i$ or eIF-2B alters the specificity of eIF-2 phosphatase. J. Biol. Chem. 259: in press, 1984.

3. Tolunay, H.E., Yang, L., Kemper, W.M., Safer, B. and Anderson, W.F.: Homologous globin cell-free transcription system with comparison of heterologous factors. Molec. Cell. Biol. 4: 17-22, 1984.

4. Tolunay, H.E., Yang, L., Anderson, W.F. and Safer, B.: Isolation of an active transcription initiation complex from HeLa cell-free extract. Proc. Natl. Acad. Sci. USA, in press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02214-07 MH

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Molecular Cloning of Eukaryotic Globin Gene Sequences

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Patricia E. Berg	Senior Staff Fellow	LMH, NHLBI
Others:	W. French Anderson	Chief	LMH, NHLBI
	Yu Gong	Visiting Fellow	LMH, NHLBI
	Donna Williams	Postdoctoral Fellow	LMH, NHLBI
	Rebecca King	Medical Technologist	LMH, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section on Molecular Cloning

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
3.4	2.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to use the mouse β-globin system as a model for eventual gene therapy of β-thalassemia in humans. Recombinant DNA technology has been applied to study two areas important for proper regulation of a gene introduced into cells: a) the effect of DNA enhancing sequences on the mouse β-globin promoter, both in tissue culture cells and in mice; and b) site specific integration of cloned genes. Various plasmids, called expression vectors, have been constructed to test the effect of known enhancers and to look for new enhancers. Attempts to isolate cellular factors which influence enhancer activity are also underway. Site specific integration of a cloned ribosomal DNA gene has been studied in cultured mouse cells where there are 300-350 copies of a homologous ribosomal gene.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

Objectives: This project has three objectives: (1) to study the effect of known enhancer sequences on expression from the mouse β-major globin gene promoter in order to maximize gene expression and examine the specificity of enhancers; (2) to detect new enhancers in order to test their effects on the mouse β-globin promoter and their cell type specificity; and (3) to determine whether site specific integration of plasmid DNA occurs at an easily detectable frequency in cultured cells.

Methods Employed:

1. To test the specificity of enhancer effects on the mouse β-globin promoter we compared the activity of two enhancers, the SV40 and the Harvey sarcoma virus (HaSV) enhancers, on the SV40 early promoter and the β-globin promoter. This was done using DNA constructions identical except for the promoter being tested. Each promoter directs expression of the *E. coli* galactokinase gene.

2. Activity of galactokinase is assayed using a transient expression assay. This involves introducing plasmid DNA into monkey kidney cells (CV-1 cells), mouse cells (L cells), or Chinese hamster cells (CH cells) in culture, then assaying galactokinase by starch gel electrophoresis or *in vitro* tube assays.

3. Using an enhancer screening system which we developed, we tested for the presence of enhancer DNA in the Rous sarcoma virus (RSV) long terminal repeat (LTR) DNA, in collaboration with Dr. Grace Ju of the Roche Institute for Molecular Biology. Different segments of the LTR were cloned into our expression vector containing the β-globin promoter and assayed using transient expression in quail cells, CV-1 cells and CH cells.

4. DNA from the LTR region of a mouse specific virus, the spleen focus forming virus (SFFV), has also been cloned into our expression vector in collaboration with Drs. Sandra Ruscetti and Linda Wolfe, NCI. This virus specifically infects erythroid cells so that enhancer activity, if present, may be highest in MEL (mouse erythroleukemia) cells.

5. Stable transformants of a *galK* negative mutant of CH cells, R1610, have been isolated containing three different plasmids, one without an enhancer, one with the SV40 enhancer and one with the HaSV enhancer. These have been analyzed for galactokinase activity and the plasmid DNA copy number was determined by DNA hybridization.

6. Plasmid DNA has been microinjected into fertilized mouse oocytes which were then implanted in pseudopregnant females in collaboration with Dr. Keith Humphries, NHLBI. The presence of the plasmid was determined by Southern blotting of DNA from the tails of the offspring. Galactokinase activity was assayed in various tissues before and after treatment with chemicals such as 5-azacytidine which is known to activate certain cloned genes.

7. Protein factors which bind to enhancer DNA are being screened for using proteins fractionated from HeLa cells and a plasmid with multiple copies of the

Methods Employed (continued):

SV40 enhancer. Binding to the ^{32}P-labelled DNA is determined by a filter binding assay.

8. To detect site specific integration, plasmids were constructed containing the thymidine kinase (TK) gene and two copies of X. borealis 5S rDNA. Mouse L TK$^-$ cells were transformed with these DNAs, with selection for the TK$^+$ gene. Transformed cells were then analyzed by Southern blot hybridization and hybridization in situ (in collaboration with Dr. Ann Henderson, Columbia University) to determine whether integration of the exogenous DNA occurred at regions of chromosomal homology, i.e., at the 5S rDNA regions.

Major Findings:

1. The mouse β-globin promoter is able to be activated up to 9.3-fold more than the SV40 promoter with the enhancers tested.

2. The presence of enhancers on our expression vector increases both galactokinase enzyme levels and the number of stable transformants in CH cells. Transformation frequency is a more sensitive indicator of enhancement than enzyme level.

3. Species specificity has been shown for the SV40 and HaSV enhancers for both the SV40 promoter and β-globin promoter in CV-1 cells, L cells and CH cells. Species specificity is also true for the RSV enhancer since it enhances better in quail cells than L or CV-1 cells (RSV is an avian virus).

4. Stable transformants of CH cells containing the galK plasmids with and without enhancers have been analyzed to determine enhancer function when integrated into host cell DNA. There is no evidence that either the SV40 or HaSV enhancer is functioning in integrated plasmids.

5. The galactokinase activity of stable transformants appears to be independent of integration site and dependent on the number of copies of the integrated plasmid.

6. Of 16 mice born after microinjection, 2 were positive for the presence of the expression vector plasmid DNA. One died at birth and the other (a male) was mated with wild type females. Germ line transmission of plasmid DNA has been observed to the fourth generation, with Mendelian segregation. Various tissues containing the plasmid DNA were negative when tested for galactokinase activity but were found to have heavily methylated DNA. Partial demethylation of this DNA was achieved by injecting the mice with a chemical, 5-azacytidine. Galactokinase levels increased only slightly with this treatment.

7. To detect site specific integration, four cell lines were analyzed by Southern blots and two of these by in situ hybridization. All four lines showed integration at different sites; the two analyzed by in situ hybridization each showed a single integration site different from the mouse L cell 5S rDNA sites.

Major Findings (continued):

Therefore, the cloned 5S rDNA did not recombine into any of the approximately 300-350 copies of the mouse 5S rDNA genes.

Significance to Biomedical Research and the Program of the Institute:

This work should increase our understanding of gene expression both at the molecular level and the organismic level. The effect of enhancer sequences on cell and tissue specific expression from the mouse β-globin promoter will be studied using known enhancers and enhancers we identify in our screening system. Identification of cellular proteins which bind to enhancer DNA will allow us to develop an *in vitro* system to study enhancers at the molecular level. The combination of these two approaches, *in vivo* and *in vitro*, should yield valuable information concerning regulation of expression of the mouse β-globin promoter. Further studies in mice using new DNA constructions should also be helpful in the eventual gene therapy of humans.

Proposed Course of the Project:

In order to better understand eukaryotic gene regulation in general and β-globin regulation specifically, studies to understand enhancer function on a molecular level will continue, with an emphasis on finding cellular factors which bind to enhancers. In addition, possible mouse specific enhancers will be tested in our screening system. Such an enhancer, if found, would be tested by microinjection of an expression vector containing this enhancer into mouse oocytes to look for tissue specific gene expression.

Publications:

1. Berg, P.E. and Anderson, W.F. Correlation of gene expression and transformation frequency with the presence of an enhancing sequence in the transforming DNA. Molec. Cell. Biol. 4: 368-370, 1984.

2. Berg, P.E., Popovic, Z., and Anderson, W.F. Promoter dependence of enhancer activity. Molec. Cell. Biol., in press.

3. Humphries, R.K., Berg, P.E., DiPietro, J., Bernstein, S., Baur, A., Nienhuis, A., and Anderson, W.F. Human and mouse globin gene sequences introduced into mice by microinjection of fertilized mouse eggs. In Kumar, A., Goldstein, A., and Vahouny, G. (Eds.): George Washington University Spring Symposia Series. III. Gene Expression, Vol. II, New York, Plenum Press, 1983, pp. 117-128.

4. Huberman, M., Berg, P.E., Curcio, M.J., DiPietro, J., Henderson, A.S., and Anderson, W.F. Fate and structure of DNA microinjected into mouse TK$^-$ L cells. Exp. Cell Res. 153: 347-362, 1984.

5. Berg, P.E., Henderson, A., Ripley, S., Yu, J-K, and Anderson, W.F. Lack of site specific recombination of exogenous DNA in mouse L cells. Biochem. Biophys. Res. Commun. 116: 959-965, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02216-05 MH

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Correction of Genetic Defects by Gene Transfer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. French Anderson	Chief	LMH, NHLBI
Others:	Sheldon Goldberg	Medical Staff Fellow	LMH, NHLBI
	Philip Kantoff	Medical Staff Fellow	LMH, NHLBI
	David Trauber	Medical Staff Fellow	LMH, NHLBI
	Daniel Kuebbing	Senior Staff Fellow	LMH, NHLBI
	Martin Eglitis	Staff Fellow	LMH, NHLBI
	Jeanne McLachlin	Visiting Fellow	LMH, NHLBI
	Judith DiPietro	Biologist	LMH, NHLBI
	Sheri Bernstein	Biologist	LMH, NHLBI

COOPERATING UNITS *(if any)*

K. Humphries, A. Nienhuis, CHB, NHLBI; S. Lewis, Research Triangle Institute, Research Triangle Park, NC; R. Popp, Oak Ridge National Laboratory, Oak Ridge, TN; E. Gilboa, Princeton University, Princton, NJ.

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.8	5.4	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods have been developed for transferring functional genes into mammalian cells in order to carry out gene therapy. Two primary techniques are: a) physical microinjection of specific cloned genes into the nucleus of individual tissue culture cells and into mouse fertilized eggs and b) use of retroviral vectors to transfer genes into tissue culture and bone marrow cells. The β-thalassemia mouse model has been used to study mechanisms involved in increased β minor globin expression.

773

Project Description:

Objectives: The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. Ultimately, the techniques would be used for attempting to correct genetic diseases in human patients.

Methods Employed:

1. Tissue culture cells are grown under standard tissue culture conditions.

2. Plasmids containing specific cloned genes are made by standard recombinant DNA techniques.

3. Gene transfer to mice by microinjection of fertilized eggs involves several specialized techniques which are briefly described below.

(a) Collection of zygotes: C57BL/6J females or C57BL-β^{thal} homozygous females are mated to LT/SV males. Females with vaginal plugs (day 1 of pregnancy) are sacrificed and fertilized eggs at the pronuclear stage are harvested from oviducts. Eggs are freed from follicle cells by mild enzyme treatment and held in special medium in a controlled atmosphere until microinjection.

(b) Microinjection and micromanipulation of eggs: Precise injection pipettes with an external diameter of 1 μ are drawn on a horizontal pipette puller. Holding pipettes of 60 to 70 μ in diameter are drawn by hand. With a Leitz inverted microscope and Leitz micromanipulators, eggs, in a small drop of media under oil, are positioned on the holding pipette so that the male pronucleus is in juxtaposition to the injection pipette. The injecting pipette is then inserted into the male pronucleus and approximately 10 pl of DNA solution is introduced.

(c) Embryo transplantation: After microinjection, zygotes are transplanted into the oviductal ampullae of day-1 pseudopregnant B6D2F1/J hybrid foster mothers. After 20 days gestation, mice are delivered spontaneously or, if necessary, by Caesarean section.

(d) Screening for donor DNA in mice: In most cases for screening, high molecular weight DNA is prepared from a small portion of tail obtained at 2 weeks of age. DNA is analyzed in Southern blots or spot blots using radioactive probes prepared from the injected sequences.

(e) Analysis of germ line transmission and gene expression: Positive mice are mated and offspring screened for evidence of germ line transmission of donor DNA sequences. Gene expression is tested in a variety of tissues using sensitive assays for protein and/or RNA. Further studies to characterize the nature of the donor DNA will include: restriction enzyme analysis to determine the methylation status and *in situ* chromosome hybridization to determine the site of integration.

Methods Employed (continued):

4. Gene transfer by retroviral vectors uses standard retroviral techniques for infection and analysis.

5. Genomic mapping is carried out by standard recombinant DNA techniques.

Major Findings:

1. The techniques necessary to successfully microinject mouse embryos are now standard. A number of genes have been transferred into eggs, the eggs brought to term in surrogate mothers, and the gene shown to be present in the living intact animal.

2. Retroviral vectors have been developed carrying the neo-r gene. These vectors are being used to infect NIH 3T3 cells in culture and mouse bone marrow cells in vivo.

3. The DNA fragment carrying the deletion has been isolated from a library of β-thalassemic mouse DNA. The fragment has been sequenced and shown to contain an unexpected insert of 68 base pairs. This insert has a sequence suggesting a possible homology to transposon-like elements found in other species.

4. Treatment of β-thalassemic mice with 5-Azacytidine results in correction of the α/β abnormality due to an increase in β^{minor} RNA synthesis. Implications of this observation are under investigation.

Significance to Biomedical Research and the Program of the Institute:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology, retrovirology, mouse genetics, embryology, and cell biology techniques to try to accomplish this goal.

Proposed Course of the Project:

Analysis of genes microinjected into mouse eggs and carried by retroviral vectors into erythroid cells will continue in order to understand regulation of the globin gene locus. Eggs from thalassemic mice are being microinjected with globin genes in an attempt to cure a genetic defect (thalassemia) in vivo.

Publications:

1. Skow, L.C., Burkhart, B.A., Johnson, F.M., Popp, R.A., Popp, D.M., Goldberg, S.Z., Anderson, W.F., Barnett, L.B., and Lewis, S.E.: A mouse model for β-thalassemia. Cell 34: 1043-1052, 1983.

2. Chiang, Y.L., Ley, T.J., Sanders-Haigh, L., and Anderson, W.F.: Human globin gene expression in hybrid 2S Mel X human fibroblast cells. Somat. Cell Molec. Genet., 1984, in press.

Publications (continued):

3. Ley, T.J., Chiang, Y.L., Haidaris, D., Anagnou, N.P., Wilson, V.L., and Anderson, W.F.: DNA methylation and regulation of the human β-like globin genes in mouse erythroleukemia cells containing human chromosome 11. Proc. Natl. Acad. Sci. USA, 1984, in press.

4. Humphries, R.K., Berg, P., DiPietro, J., Bernstein, S., Baur, A., Nienhuis, A.W., and Anderson, W.F.: Non expressed globin gene sequences introduced into mice by microinjection are heavily methylated. Br. J. Haematol., 1984, in press.

Annual Report of the
Section on Laboratory Animal Medicine and Surgery, Surgery Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1983 to September 30, 1984

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various small animal species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species are maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3, 14-E and 28.

The animal surgery laboratory located in Building 14-E supported studies for investigative staff in the Cardiology Branch, Clinical Hematology Branch, Hypertension-Endocrine Branch, Laboratory of Chemistry, Laboratory of Kidney and Electrolyte Metabolism, Laboratory of Technical Development, Molecular Disease Branch, and the Surgery Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological specimens. The laboratory operates an x-ray catheterization suite, blood analysis laboratory, sterile operating suites, and special study suites required to meet IR requirements.

The NHLBI Sheep Colony continues year-round breeding of laboratory sheep. More than 550 animals were delivered to laboratories meeting requirements of gestation stages from 120-140 days and various age and size lambs, young adults, and aged sheep. In addition, postoperative animal models have been maintained at the colony, 10-15 units of blood has been delivered every 2-3 weeks for use in cardiopulmonary by-pass studies and more than 30 tons of feed supplies have been delivered to NIH to allow continued feeding of similar feed rations to sheep maintained for biomedical research studies.

Laboratory studies have been underway to define hemodynamic parameters and tissue morphology resultant of hereditary left ventricular hypertrophy due to infracoronary left ventricle outflow tract obstruction. Disease processes will be characterized to determine usefulness of this unique animal model for future laboratory study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03401-08 LAMS

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Newfoundland Breeding Colony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. E. Pierce	Chief	SLAMS, SB, ODIR, NHLBI
Others:	M. Jones	Senior Surgeon	SB, NHLBI

COOPERATING UNITS *(if any)*
Clinic of Surgery Z01 HL 02697-05 SU

LAB/BRANCH
Surgery Branch

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)
- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Newfoundland Breeding Colony has been developed as a source of laboratory dogs affected with left ventricular hypertrophy (LVH) due to infracoronary left ventricle outflow tract obstruction and other spontaneously occuring heart defects. All have some form of hereditary subaortic stenosis (SAS) and/or pulmonary outflow tract obstruction.

778

Project Description:

Availability of a naturally occurring animal model for study of LVH resultant of infracoronary LV outflow tract obstruction is important because technical difficulties have not been satisfactorily overcome in attempts to produce such a defect in normal animals.

Discrete subaortic stenosis has been studied in Newfoundland dogs at the School of Veterinary Medicine, University of Pennsylvania where initial breeding experiments suggested that it is inherited and either a polygenic or an autosomal dominant trait with modifiers.

More than 60 dogs have been maintained with monitoring of hemodynamics using cardiac catherazation and ultrasound techniques.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03402-08 LAMS

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NHLBI Laboratory Sheep Colony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Joseph E. Pierce, D.V.M., Chief, SLAMS, SB, ODIR, NHLBI

COOPERATING UNITS *(if any)*

1. Laboratory of Developmental Neurobiology, IRP, NICHD
2. VRB, DRS

LAB/BRANCH

Surgery Branch

SECTION

Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Laboratory Sheep Colony is an NIH animal resource providing varied age sheep that meet specific year-round requirements of the Laboratory of Kidney and Electrolyte Metabolism, Laboratory of Technical Development and the Surgery Branch, DIR, NHLBI; and the Laboratory of Developmental Neurobiology, IRP, NICHD. Maintenance regimens in use have resulted in successful year-round breeding and production of healthy varied age sheep.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commercial sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

780

Project Description

The breeding colony continues as a source of sheep for the Surgery Branch, Laboratory of Technical Development, and Laboratory of Kidney and Electrolyte Metabolism meeting requirements of young lambs and pregnant ewes as required. From 730 to 850 animals were maintained to allow delivery of more than 550 sheep to NIH and other facilities for laboratory use during the report period.

The contractor has been responsible for developing and updating husbandry techniques as instructed by the project officer that allow optimal conditions for natural year-round breeding at the contract site. The project officer has been responsible for monitoring techniques and updating guidelines followed by the contractor to meet NIH laboratory requirements along with animal health regimes (sheep property of NIH) that allow minimal susceptability of all age groups to common sheep diseases.

Immunization protocols direct personnel to administer specific toxoids and bacterins to lambs with bi/tri-weekly boosters of each by the 6th week of age. This practice has essentially eliminated enterotoxemia and more than 90% of chronic pneumonia previously experienced. Repeated administration of indicated biologicals is carried out in all age groups at designated periods of development and production.

The necessity of an accurate method of pregnancy diagnosis during early gestation was determined during initial development of the colony. Natural seasonal and environmental conditions effect the conception rate varying from 0-100%. Lindahl's technique using Doppler ultrasound with rectal examination has been adequate. It allows accurate diagnosis of up to 100% of pregnant sheep from 21-35 days gestation. Examinations are performed weekly by contract personnel with more than 1,500 examinations completed per year to detect approximately 500 pregnant ewes conceived over a 52-week period.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor internal parasite infestation by random fecal sampling from various animal groups with microscopic examination using direct and flotation methods. CBC and blood chemistry profiles are performed on animals delivered to the laboratory to monitor health and nutrition status. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

In addition to producing sheep for laboratory use, the contractor has maintained animals following surgical procedures for periods over 6 months and returned for study.

Also, 10-20 units of blood has been collected in CPD bags every 2-3 weeks for use in cardiopulmonary by-pass studies in NHLBI laboratories.

More than 30 tons of feed supplies were delivered to NIH to allow continuation of colony animal diets in laboratory facilities.

This project will continue as IR and other NIH programs have sufficient requirements that justify the continued support of this laboratory sheep resource. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information

Contract Number:	263-80-C-0007 - approximately $266,000 - 10/1/83-9/30/84
Contract Site:	White House Farms, Inc. Rt. 1, Box 403-E Luray, Virginia 22835
PI:	Max Foltz, Contractor Rick Miller, Colony Manager
Total Manyears:	8.0
Professional:	2.0
Other:	6.0

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 through September 30, 1984

This is the final report of the Section on Theoretical Biophysics. The primary interest of the section has been the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal, and membrane physioloy. The section has been concerned both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Much of the research of the section has centered on the mechanism of urine fomation in the mammalian kidney and on theoretical aspects of solute and water transport in epithelial structures.

During the past year, areas of work have included: (1) The thermodynamic and kinetic analysis of transport processes, (2) the qualitative analysis of equations describing kidney models, (3) the development of analytical solutions of kidney models, (4) the development and theoretical analysis of numerical methods, and (5) the computer simulation of renal function.

In earlier studies it was found that the solution manifolds of kidney models have multiple solution branches; research on this problem has continued. A detailed study of the solutions of a four tube model of the renal medulla has been published (Garner, Kellogg, and Stephenson, Math. Biosciences 65:125-150 (1983)). The results include an existence theorem for solutions of the equations describing the model, the derivation of an exact solution for a limiting form of the model equations, and numerical studies of the exact solution. These showed that the solution manifold is folded into three sheets: an upper and lower stable sheet, and a middle unstable sheet. The upper sheet consists of highly concentrating solutions to the model equations, and on this sheet the concentration ratio is determined by the dissipative loss of solute caused by inefficient vascular exchange. With no loss, the upper sheet is not present, and the solution manifold contains at most two sheets (Garner, Kellogg, and Stephenson).

Qualitatively similar results have been obtained and published for a multinephron, multisolute model of the mammalian kidney (Mejia and Stephenson, Math. Biosciences 68:279-298 (1984)). In this paper a method of numerically solving the differential equations describing solute and water flow in the model by a combination of Newton and continuation techniques is described. This method is used to generate a connected component of the steady state solution manifold of the model. As in the simple four-tube model, a three dimensional section of this manifold is shown to be convoluted with upper and lower sheets of stable solutions connected by an unstable middle sheet. Two

dimensional sections of this surface are followed from a trivial constant profile of concentrations in the nonconcentrating kidney to the profiles of the maximally concentrating kidney. Study of these sections shows that for a given choice of model parameters there may exist no solution, there may be a unique solution, or there may be multiple solutions. A study of the time dependent solutions shows that the dynamic transition from the lower to the upper state and return may be via a hysteresis loop (Mejia and Stephenson).

Further studies have been carried out on the effect of various parameter modifications on the concentrating mechanism. These studies have used primarily a central core model of the renal medulla. This model utilizes CONKUB, the conversational pathfollower, to follow the effect of the changes. Anatomically the model includes short and long nephrons discharging into a common collecting duct and the renal pelvis. These studies have verified that with an idealized set of parameters it is possible to generate a significant concentration gradient in the inner medulla with no active solute transport out of the thin ascending limb of Henle's loop. When experimental values are substituted for the idealized set, there is substantial flattening of the inner medullary concentration profiles. This we interpret as being primarily caused by the increased urea permeability of the loop of Henle. This decreases water extraction from the descending limb of the loop of Henle and, as a consequence, salt diffusion out of the thin ascending limb. Appropriate adjustment of the water and urea permeabilities of the inner medullary collecting ducts and of the epithelium separating the renal pelvis from the papilla gives interstitial profiles of salt and urea that are consistent with those experimentally observed. The medullary collecting duct is not, however, completely osmotically equilibrated with the interstitium. These studies have given us much greater insight into the various factors affecting the concentration of urine, but so far we do not have a theoretical model of the concentrating process in the inner medulla that is entirely consistent with the experimental observations. These numerical studies are now being written up for publication (Kaimal, Mejia, and Stephenson).

A single nephron model for the transport of p-aminohippurate (PAH) and its analogues through the kidney has been investigated and the results published (Kaimal, Kellogg, and Stephenson, Math. Biosciences 69:103-129 (1984)). One of the new features of the model is that it incorporates the epithelial cells surrounding the proximal tubules. These cells are the main anatomical sites of transport of PAH. The kinetics of PAH transport is described by a set of linear conservation equations. A stable numerical scheme that is backward Euler in both space and time coordinates is used to analyze the mathematical problem. We obtain results that are compatible with those of previously published models and with experimental data. An advantage of the present model is

that it could be used to evaluate radionuclide images in terms of transport coefficients, flow rates, etc. Thus, these imaging techniques could be used to evaluate renal function in a more meaningful manner (Kaimal, Kellogg, and Stephenson).

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung. and Blood Institute
October 1, 1983 through September 30, 1984

Investigations during the above time period, as in previous years, centered primarily on studies of coronary, valvular, myocardial, congenital, and miscellaneous heart diseases. Additionally, the Ultrastructure Section was involved in many experimental studies and had several studies in collaboration with the Pulmonary Branch.

CORONARY HEART DISEASE

Sudden death is the most frequent mode of death in patients with coronary heart disease. We examined the hearts at necropsy in 70 victims aged 21 to 81 years (mean 50) with sudden coronary death (SCD), all of whom had been examined at necropsy. In each patient, each of the 4 major coronary arteries were excised intact and cut into 5-mm segments and the amount of cross-sectional area (XSA) narrowing in each 5-mm segment was examined. In general, the right coronary artery is 10 cm long, left anterior descending 10, left main 1, and left circumflex usually about 6 cm. Thus approximately 27 cm of coronary artery was available for examination from each patient. Since each 1 cm is divided into 2 five-mm segments, approximately 50 to 55 histologic sections were examined from each patient. Of 3,484 five-mm segments examined from the 4 major arteries, 950 (27%) were narrowed 76-100% in XSA; 1127 (32%), 51-75%; 689 (20%), 26-50%, and 718 (21%), 0-25%. More severe narrowing occurred in the proximal compared to the distal halves of the left anterior descending, left circumflex and right coronary arteries. Comparison between the 31 previously symptomatic victims to the 39 victims who had previously been asymptomatic disclosed a significantly higher mean percent of severely narrowed 5-mm segments and a lower mean percent of minimally narrowed segments in the symptomatic group. Comparison of the 31 patients who had healed myocardial infarcts at necropsy to the 31 who did not disclosed a higher mean percent of 5-mm segments narrowed severely and a lower mean percent of segments narrowed minimally in those with a left ventricular scar. Comparison between victims whose hearts weighed over 450 grams to those weighing less disclosed a higher mean percent of severely narrowed segments and a lower mean percent of minimally narrowed segments in the patients with the larger hearts.

Among the 70 victims of SCD, certain clinical and morphologic findings in the 13 with a coronary thrombus were compared to those in the 57 without a thrombus. Compared to those without, the 13 with thrombus were younger, had a lower mean percent of XSA narrowing by plaque at the site of maximal coronary stenosis, and had a higher mean percent of 5-mm segments of the 4 major epicardial coronary arteries minimally narrowed by plaque. No differences occurred in the 2 groups with regards to sex, previous angina pectoris and/or clinical acute myocardial infarction, healed myocardial infarcts at necropsy, mean heart weight, number of major coronary arteries severely narrowed by plaque or the mean percent of 5-mm segments of the 4

major coronary arteries narrowed 76-100% in XSA by plaque. Thus, coronary thrombi are infrequent in victims of SCD and when observed there significance is uncertain because victims of SCD without coronary thrombi have similar amounts of severe narrowing.

In a subset of the above studied patients, 60 male victims of SCD aged 31-70 years were also analyzed to see if differences in the amounts the coronary narrowing occurred in 4 aged decades. Of 2995 five-mm segments examined of the 4 major coronary arteries, no significant difference were observed in the mean percent of 5-mm long segments narrowed severely (76-100% in XSA) in each decade. Thirty-three percent in the 5 patients aged 31-40 years, 29% in the 27 patients aged 41 to 50 years, 27% in the 18 patients aged 51-60 years and 30% in the 10 patients aged 61-70 years. Thus, as groups, both young and old victims of SCD have similar amounts of severe coronary narrowing but the younger victims have a greater proportion of their major coronary arteries minimally narrowed.

VALVULAR HEART DISEASE

To assess the reliability of M-mode echocardiographic patterns of mitral valve prolapse (MVP) (echo MVP) in detection of morphologic evidence of MVP (morphologic MVP), operatively excised mitral valves and corresponding M-mode echocardiograms from 65 patients with chronic, severe, isolated, pure mitral regurgitation (MR) were studied. Of the 65 patients, 45 (69%) had echo MVP (either holosystolic or mid-to-late systolic prolapse patterns on preoperativ M-mode echocardiogram) and 42 (93%) of them had morphologic MVP; of the 3 without morphologic MVP, 2 had ruptured chordae tendineae from infective endocarditis and 1 had papillary muscle dysfunction from atherosclerotic coronary heart disease. Of the 20 patients without echo MVP, 14 (70%) had no morphologic MVP (9 had papillary muscle dysfunction from coronary heart disease, 4 had infective endocarditis on previous normal valves and 1 had rheumatic heart disease). Of the 48 patients with morphologic MVP, 42 (88%) had echo MVP and most had considerably dilated mitral anulae; the other 6 had ruptured chordae tendineae with less degrees of anular dilatation. Of the 17 patients without morphologic MVP, 3 had echo MVP; of the 14 with neither echo nor morphologic MVP, 9 had papillary muscle dysfunction from coronary artery disease, 4 had infective endocarditis on previously normal valves and 1 had rheumatic heart disease. The patients with very dilated mitral anuli and leaflet areas generally had holosystolic (hammocking) patterns on echo; the patients with small anuli and leaflet areas usually had mid-to-late systolic (buckling) prolapse patterns.

The degree of XSA narrowing by atherosclerotic plaque in each of the 4 major epicardial coronary arteries (right, left main, left anterior descending and left circumflex) was determined at necropsy in 37 patients (30 men and 7 women) aged 34 to 77 years (mean 54) with severe, isolated chronic, pure aortic regurgitation (AR). In 7 patients (19%), $\geq$1 major coronary artery was narrowed 76-100% in XSA at some point. Of the 148 major coronary arteries examined in the 37 patients, 12 arteries (8%)

were narrowed at some point 76-100% in XSA. Each of the 148 major coronary arteries were divided into 5-mm-long segments (average 53 per patient) and a histologic section from each segment was examined. Of the 1,977 segments, 1,087 were narrowed 0 to 25%, 669 (34%) 26 to 50%, 170 (9%) 51 to 75%, 48 (2%) 76 to 95% and 3 (0.001%) 96 to 100%. The average amount of XSA narrowing by atherosclerotic plaque per segment was about 28%. Of the 37 patients, 9 had had angina pectoris, 2 of whom had significant (>75% XSA reduction) coronary narrowing; 2 other patients had had acute myocardial infarction clinically, 1 of whom had significant coronary narrowing at necropsy. Thus, in general, the amount of coronary narrowing in our 37 adults with severe, pure, isolated, chronic AR was relatively mild.

Among patients with mitral stenosis, calcific deposits in the mitral valve have been reported to be infrequent in persons with mitral stenosis living in parts of the world where the average total serum cholesterol is low, i.e. less than 150 mg/dl. To see if a relation existed between calcific deposits in stenotic mitral valves and the total cholesterol level we analyzed 155 patients with rheumatic mitral stenosis in whom the operatively excised mitral valve was x-rayed to determine the presence and extent of calcific deposits and also the preoperative level of total serum cholesterol. The amount of mitral calcium was graded 0 to 4+ and the average cholesterol for each of the 5 groups was as follows: 0 deposits - 21 patients (14%) (TC = 188 mg/dl); 1+ - 50 patients (32%) (TC = 196 mg/dl); 2+ - 22 patients (14%) (TC = 198 mg/dl); 3+ - 37 patients (24%) (TC = 205 mg/dl), and 4+ - 25 patients (16%) (TC = 184 mg/dl). These average values of TC and the mean ages of the patients in each of the 5 groups of mitral calcium were not significantly different.

The St. Jude Medical (SJM) prosthesis is increasingly being used for cardiac valve replacement. We examined the hearts of 2 patients who died early after replacement of the aortic valve with SJM prosthesis and in each it appeared that movement of 1 of 2 prosthetic leaflets was interferred with by underlying severely hypertrophied myocardium and leftward positioning of the ventricular septum or by severely thickened anterior mitral leaflet. Thus, we cautioned the use of this prosthesis in old patients with aortic stenosis because these individuals have the aorta moved in a rightward direction so that the prosthesis is inserted over a good portion of the ventricular septum, an occurrence not occurring in younger people.

CONGENITAL HEART DISEASE

Little information is available on results following replacement of the tricuspid valve or tricuspid valve annuloplasty in patients with Ebstein's anomaly of the tricuspid valve. We analyzed 6 patients who had both pre and postoperative cardiac catheterization following an operation on the tricuspid valve for severe tricuspid regurgitation due to Ebstein's anomaly. The operations in each of the 6 patients were associated with a decrease in symptoms of cardiac dysfunction, a decrease in cardiac size, an increase in cardiac index and an increase in right ventricular and right atrial pressures.

Observations are described in 12 massively (>300 pounds) obese patients aged 25 to 59 years (mean 37) (5 women) weighing 312 to >500 pounds (mean 381): 7 had had systemic hypertension; 4, hypersomnia or sleep apnea; 2, diabetes mellitus, and 1, symptomatic coronary artery disease. Five died suddenly from undetermined causes; 2, from right-sided congestive heart failure; 1, from acute myocardial infarction; 1, from aortic dissection; 1, from intracerebral hemorrhage; 1, from a drug overdose, and 1, postoperatively shortly after an ileal bypass. The heart weight was increased in all 12 patients. The heart weight to body weight ratio expressed as a percent ranged from 0.22 to 0.61 (mean 0.37) (normal for men = 0.42 to 0.46 [mean 0.43]; for women = 0.38 to 0.46 [mean 0.40]). The left ventricular cavity was dilated in 11 patients, and the right ventricular cavity, in 12. Only 2 patients (aged 42 and 59 years) had 1 or more major epicardial coronary arteries narrowed >75% in XSA by atherosclerotic plaque, 1 of whom had no symptoms of myocardial ischemia. Of 664 five-mm segments from the 4 major epicardial coronary arteries from 11 patients (mean 60 per patient), 431 (65%) were narrowed 0-25% in XSA; 143 (21%), 26-50%; 73 (11%), 51-75%, and 17 (3%), 76-100%. Thus, these extremely obese patients who died prematurely did not have more coronary atherosclerosis than might be expected for their ages.

Because QT-interval prolongation (QTIP) has been associated with sudden death in patients using liquid protein diets, the role of QTIP was investigated in 3 women, aged 29, 32, and 37 years, with anorexia nervosa in whom both necropsy and electrocardiograms were available. At death, they weighed 41.8, 23.5,, and 31.0 kg, respectively. Electrocardiogram recorded <7 days before death in each showed prolonged QT-intervals: 0.61, 0.47, and 0.46 sec, respectively, when corrected for heart rate. Terminal ventricular tachyarrhythmias were documented in 2 patients, including torsade de pointes in 1. The clinical and necropsy findings in these 3 patients provide evidence that sudden death in anorexia nervosa may result from QTIP and indicate the need for electrocardiographic monitoring of such patients.

Acquired immune deficiency syndrome (AIDS), a recently described disease affecting primarily homosexuals and intravenous drug abusers, was studied in 18 necropsy patients. Five (28%) had abnormalities in the heart and in each they consisted of focal deposits of Kaposi's sarcoma. None of the cardiac lesions, however, produced evidence of cardiac dysfunction.

There has never been a study comparing quantitatively publications in the 7 major English language cardiology journals. Such was done for the year 1983 for 7 major cardiac journals. In each the number of pages, types of article, figures and tables published and numbers of authors per article were analyzed. Although systemic hypertension is present in about 25 million Americans only 2% of the 2,250 articles published in the regular issues of the 7 English language cardiovascular journals concerned systemic hypertension.

Most hearts contain adipose tissue on their surfaces and this tissue tends to increase with age and with body weight. Certain clinical and morphologic findings were described in 55 patients whose hearts at autopsy contained so much fat that they floated in water. The patients were 47 to 89 years old (mean 67). Symptomatic coronary heart disease was present in 28 (51%) and valvular heart disease (mitral stenosis) in 3 (5%). The heart at necropsy was enlarged (>350 g for women and >400 g for men) in 45 patients (82%). The mean heart weight for the 31 women was 470 g and for the 14 men, 515 g. In addition to the severe increase in fat in the atrioventricular sulci and over both ventricles, the amount of fat in the atrial septum was increased in all patients. In 14 patients (25%), the thickness of the atrial septum cephaled to the fossa ovale was $\geq$2 cm. Excessive fat in this location is called "lipomatous hypertrophy of the atrial septum." Of the 16 patients (29%) with fatal acute myocardial infarction, 7 (44%) had rupture of either the left ventricular free wall or ventricular septum. The high frequency of cardiac rupture in these patients supports the contentiion that rupture during acute myocardial infarction is more common in the fatty than in the non-fatty heart.

A simple technique to aid in the interpretation of the cardiac and aortic silhouette on chest roentgenogram was described. It utilized a 3 x 5 inch card cut in a square. This little card can be carried in a physician's pocket or purse so that when a chest x-ray is examined each of the various cavities and great vessels can be more systematically examined.

ULTRASTRUCTURE STUDIES

Recent electron microscopic observations on cardiac structure in hypertrophy were reviewed and summarized, with emphasis on: 1) structural-functional correlations during the evolving progression of cardiac hypertrophy, and 2) the concept that cardiac hypertrophy involves not only an increase in heart size, but also progressive, initially subtle, structural changes in the muscle cells.

The cardiac ultrastructural findings in idiopathic restrictive cardiomyopathy were found to be hypertrophy and mild disarray of myocytes, thickening of the endocardium and interstitial fibrosis. The arrangement of the fibrous tissue in myocardial interstitium was tangled, suggesting that it may interfere with proper relaxation of myocardium during diastole. Thus, these observations may have a relation to the functional abnormalities of myocardial restriction.

Several studies were made of different aspects of the chronic cardiomyopathy produced by antineoplastic agents of the anthracycline type (doxorubicin and daunorubicin). The clinical and pathologic features of this cardiomyopathy were reviewed together with the mechanisms by which this lesion is considered to develop. Data obtained in collaboration with Dr. Eugene Herman of the FDA were presented to show that the severity of anthracycline cardiomyopathy is markedly reduced by the concurrent

administration of ICRF-187, a compound that appears to act by interfering with the formation of cytotoxic free radicals that produce myocardial damage. The formation of these free radicals is thought to be mediated by a complex that forms between anthracyclines and iron. In another study, ICRF-187 was found to protect against the hepatotoxic damage produced by acetaminophen, a model system in which the lesion is also thought to result from iron-mediated free radical formation.

The potential protection by vitamin E against anthracycline cardiotoxicity was evaluated in miniature swine. The degree of protection observed was minimal. A high degree of cardioprotection was obtained by giving doxorubicin in the form of drug-containing liposomes (as opposed to the free drug in solution) to beagle dogs. It remains to be determined whether this technique of administration results in effective antineoplastic therapy.

There were several studies on pulmonary ultrastructure. Studies were made of the structure of elastic fibers in lung, muchal ligament and aorta, using light and electron microscopic techniques for the localization of elastin by means of ferritin- or peroxidase-labeled antielastin antibody. The use of these techniques permitted identification of elastic fibers during embryonic development at a stage in which the fibers cannot be definitely identified by means of routine ultrastructural staining methods. Antielastin antibody was also used to study structural alterations in elastase-treated elastic fibers, because such fibers are extremely difficult to stain properly. A review was prepared of the techniques, applications, results and diagnostic implications of pulmonary bronchoalveolar lavage, a technique which has been extensively used by the Pulmonary Branch in the diagnosis and serial evaluation of patients with interstitial lung disorders. In collaboration with Dr. Francoise Basset and associates in Paris, a study was made of the applicability of the immunodetection of OKT6-positive cells in bronchoalveolar lavage fluid for the diagnosis of histiocytosis X.

Several clinicopathologic and electron microscopic studies were made to characterize the myocardial and skeletal muscle lesions produced by monensin in cattle and swine. The monensin model represents a unique system for the study of cardiac damage produced directly by experimentally induced alterations in sodium transport.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03796-01 PA

PERIOD COVERED
October 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Sudden Coronary Death: Relation of Amount and Distribution of Coronary Narrowing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

The amount and distribution of coronary arterial narrowing by atherosclerotic plaque at necropsy is described in 70 victims aged 22-81 years (mean 50) of sudden coronary death (SCD). Of 3484 five-mm segments examined (mean 50 per patient) from the 4 major (left main [LM], left anterior descending [LAD], left circumflex [LC] and right) coronary arteries, 950 (27%) were narrowed 76-100% in cross-sectional area (XSA); 1127 (32%), 51-75%; 689 (20%), 26-50%, and 718 (21%), 0-25%. More extensive severe narrowing occurred in the proximal compared to the distal halves of the LAD, LC and right coronary arteries. Comparison between the 31 previously symptomatic victims (either angina pectoris and/or a clinical acute myocardial infarction [MI]) to the 39 victims who had previously been asymptomatic disclosed a significantly higher mean percent of severely (76-100% XSA) narrowed 5-mm segments (30%-vs-25% [$p<.005$]) and a lower mean percent of minimally (0-25% XSA) narrowed segments in the symptomatic group (15%-vs-25%, $p<.001$). Comparison of the 31 patients who had a healed MI at necropsy to the 39 patients who did not disclosed a higher mean percent of 5-mm segments narrowed 76-100% in XSA (33%-vs-24%, $p<.001$) and a lower mean percent of segments narrowed minimally in those with a left ventricular scar (13%-vs-26%, $p<.001$). Comparison between victims whose hearts weighed >450 gms to those weighing ≤450 gms disclosed a higher mean percent of severely narrowed segments (19%-vs-23%, $p<.01$) and a lower mean percent of minimally narrowed segments (29%-vs-24%, $p<.005$) in the large-heart group.

792

Project Description:

The amount and distribution of coronary arterial narrowing by atherosclerotic plaque at necropsy is described in 70 victims aged 22-81 years (mean 50) of sudden coronary death (SCD). Of 3484 five-mm segments examined (mean 50 per patient) from the 4 major (left main [LM], left anterior descending [LAD], left circumflex [LC] and right) coronary arteries, 950 (27%) were narrowed 76-100% in cross-sectional area (XSA); 1127 (32%), 51-75%; 689 (20%), 26-50%, and 718 (21%), 0-25%. More extensive severe narrowing occurred in the proximal compared to the distal halves of the LAD, LC and right coronary arteries. Comparison between the 31 previously symptomatic victims (either angina pectoris and/or a clinical acute myocardial infarction [MI]) to the 39 victims who had previously been asymptomatic disclosed a significantly higher mean percent of severely (76-100% XSA) narrowed 5-mm segments (30%-vs-25% [$p<.005$]) and a lower mean percent of minimally (0-25% XSA) narrowed segments in the symptomatic group (15%-vs-25%, $p<.001$). Comparison of the 31 patients who had a healed MI at necropsy to the 39 patients who did not disclosed a higher mean percent of 5-mm segments narrowed 76-100% in XSA (33%-vs-24%, $p<.001$) and a lower mean percent of segments narrowed minimally in those with a left ventricular scar (13%-vs-26%, $p<.001$). Comparison between victims whose hearts weighed >450 gms to those weighing $\leq$450 gms disclosed a higher mean percent of severely narrowed segments (19%-vs-23%, $p<.01$) and a lower mean percent of minimally narrowed segments (29%-vs-24%, $p<.005$) in the large-heart group.

Publication:

Warnes, C. A., and Roberts, W. C.: Sudden Coronary Death: Relation of Amount and Distribution of Coronary Narrowing at Necropsy to Previous Symptoms of Myocardial Ischemia, Left Ventricular Scarring and Heart Weight. American Journal of Cardiology, 54:65-73, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03797-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Sudden Coronary Death: Comparison of Patients with to Those Without Coronary

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS: 416 hours	PROFESSIONAL 416 hours	OTHER

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Among 70 victims of sudden coronary death (SCD), certain clinical and morphologic findings in the 13 with a coronary thrombus are compared with the findings in 57 victims without a coronary thrombus. Compared to those without, the 13 with a thrombus were younger (mean age 43 - vs - 51 years, $p = <0.02$); had a lower mean percent of cross-sectional area (XSA) narrowing by plaque at the site of maximal coronary stenosis (89% - vs - 95%, $p = <0.01$); and had a higher mean percent of 5-mm segments of the 4 major epicardial coronary arteries minimally narrowed (0-25% in XSA) by plaque (27% - vs - 19%, $p = <0.001$). No differences occurred in the 2 groups with regard to sex, previous angina pectoris and/or clinical acute myocardial infarction, healed myocardial infarction at necropsy, mean heart weight, number of major coronary arteries narrowed 76-100% in XSA by atherosclerotic plaque, or the mean percent of 5-mm segments of the 4 major epicardial coronary arteries narrowed 76-100% in XSA by atherosclerotic plaque. Thus, coronary thrombi are infrequent in victims of SCD, and when observed, their significance is uncertain because victims of SCD without coronary thrombi have similar amounts of severe coronary narrowing.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Among 70 victims of sudden coronary death (SCD), certain clinical and morphologic findings in the 13 with a coronary thrombus are compared with the findings in 57 victims without a coronary thrombus. Compared to those without, the 13 with a thrombus were younger (mean age 43 - vs - 51 years, $p = <0.02$); had a lower mean percent of cross-sectional area (XSA) narrowing by plaque at the site of maximal coronary stenosis (89% - vs - 95%, $p = <0.01$); and had a higher mean percent of 5-mm segments of the 4 major epicardial coronary arteries minimally narrowed (0-25% in XSA) by plaque (27% - vs - 19%, $p = <0.001$). No differences occurred in the 2 groups with regard to sex, previous angina pectoris and/or clinical acute myocardial infarction, healed myocardial infarction at necropsy, mean heart weight, number of major coronary arteries narrowed 76-100% in XSA by atherosclerotic plaque, or the mean percent of 5-mm segments of the 4 major epicardial coronary arteries narrowed 76-100% in XSA by atherosclerotic plaque. Thus, coronary thrombi are infrequent in victims of SCD, and when observed, their significance is uncertain because victims of SCD without coronary thrombi have similar amounts of severe coronary narrowing.

Publication:

Warnes, C. A., and Roberts, W. C.: Sudden Coronary Death: Comparison of Patients With to Those Without Coronary Thrombus at Necropsy. American Journal of Cardiology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03798-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Comparison at Necropsy by Age Group of Amount and Distribution

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A comparison of the amount and distribution of coronary arterial narrowing by atherosclerotic plaque at necropsy is described in each of 4 decades (31-70 years) of 60 male victims of sudden coronary death (SCD). Of 2995 five-mm segments examined (mean 50 per patient) of the 4 major (left main [LM], left anterior descending [LAD], left circumflex [LC] and right) coronary arteries, no significant differences were observed in the mean percent of 5-mm long segments narrowed severely (76-100% in cross-sectional area [XSA]) in each decade: 33% in the 5 patients aged 31-40 years; 29% in the 27 patients aged 41-50 years; 26% in the 18 patients aged 51-60 years, and 30% in the 10 patients aged 61-70 years. The mean percent of 5-mm segments severely narrowed was significantly ($p < .05$) greater in the proximal halves than in the distal halves of the LAD, LC and right coronary arteries in only the 2 older decades. The mean percent of 5-mm segments minimally (0-25%) narrowed, however, was significantly higher in the younger than in the older decades. Thus, as groups, both young and old victims of SCD have similar amounts of severe coronary narrowing, but the younger victims have a greater proportion of their major coronary arteries minimally narrowed.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description:

A comparison of the amount and distribution of coronary arterial narrowing by atherosclerotic plaque at necropsy is described in each of 4 decades (31-70 years) of 60 male victims of sudden coronary death (SCD). Of 2995 five-mm segments examined (mean 50 per patient) of the 4 major (left main [LM], left anterior descending [LAD], left circumflex [LC] and right) coronary arteries, no significant differences were observed in the mean percent of 5-mm long segments narrowed severely (76-100% in cross-sectional area [XSA]) in each decade: 33% in the 5 patients aged 31-40 years; 29% in the 27 patients aged 41-50 years; 26% in the 18 patients aged 51-60 years, and 30% in the 10 patients aged 61-70 years. The mean percent of 5-mm segments severely narrowed was significantly ($p < .05$) greater in the proximal halves than in the distal halves of the LAD, LC and right coronary arteries in only the 2 older decades. The mean percent of 5-mm segments minimally (0-25%) narrowed, however, was significantly higher in the younger than in the older decades. Thus, as groups, both young and old victims of SCD have similar amounts of severe coronary narrowing, but the younger victims have a greater proportion of their major coronary arteries minimally narrowed.

Publication:

Warnes, C. A. and Roberts, W. C.: Comparison at Necropsy by Age Group of Amount and Distribution of Narrowing by Atherosclerotic Plaque in 2995 five-mm Long Segments of 240 Major Coronary Arteries in 60 Men Aged 31-70 Years with Sudden Coronary Death. American Heart Journal, in press, September 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03799-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Frequency and Significance of M-mode Echocardiographic Evidence of Mitral Valve

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Bruce F. Waller, Senior Staff Fellow, Pathology Branch, NHLBI
Barry J. Maron,

Albert A. Del Negro, Department of Medicine (Cardiology), Georgetown University Medical Center, Washington, D.C.
John S. Gottdiener,
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 years	416 years	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To assess the reliability of M-mode echocardiographic patterns of mitral valve prolapse (MVP) (echo MVP) in detection of morphologic evidence of MVP (morphologic MVP), operatively excised mitral valves and corresponding M-mode echocardiograms from 65 patients with chronic, severe, isolated, pure mitral regurgitation (MR) were studied. Of the 65 patients, 45 (69%) had echo MVP (either holosystolic or mid-to-late systolic prolapse patterns on preoperative M-mode echocardiogram) and 42 (93%) of them had morphologic MVP; of the 3 without morphologic MVP, 2 had ruptured chordae tendineae from infective endocarditis and 1 had papillary muscle dysfunction from atherosclerotic coronary heart disease. Of the 20 patients without echo MVP, 14 (70%) had no morphologic MVP (9 had papillary muscle dysfunction from coronary heart diesease, 4 had infective endocarditis on previous normal valves and 1 had rheumatic heart disease). Of the 48 patients with morphologic MVP, 42 (88%) had echo MVP and most had considerably dilated mitral anulae; the other 6 had ruptured chordae tendineae with less degrees of anular dilatation. Of the 17 patients without morphologic MVP, 3 had echo MVP (coronary artery disease in 1 and infective endocarditis on a previous normal valve in 2); of the 14 with neither echo nor morphologic MVP, 9 had papillary muscle dysfunction from coronary artery disease, 4 had infective endocarditis on previously normal valves and 1 had rheumatic heart disease. The patients with very dilated mitral anuli and leaflet areas generally had holosystolic (hammocking) patterns on echo; the patients with small anuli and leaflet areas usually had mid-to-late systolic (buckling) prolapse patterns.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

To assess the reliability of M-mode echocardiographic patterns of mitral valve prolapse (MVP) (echo MVP) in detection of morphologic evidence of MVP (morphologic MVP), operatively excised mitral valves and corresponding M-mode echocardiograms from 65 patients with chronic, severe, isolated, pure mitral regurgitation (MR) were studied. Of the 65 patients, 45 (69%) had echo MVP (either holosystolic or mid-to-late systolic prolapse patterns on preoperative M-mode echocardiogram) and 42 (93%) of them had morphologic MVP; of the 3 without morphologic MVP, 2 had ruptured chordae tendineae from infective endocarditis and 1 had papillary muscle dysfunction from atherosclerotic coronary heart disease. Of the 20 patients without echo MVP, 14 (70%) had no morphologic MVP (9 had papillary muscle dysfunction from coronary heart diesease, 4 had infective endocarditis on previous normal valves and 1 had rheumatic heart disease). Of the 48 patients with morphologic MVP, 42 (88%) had echo MVP and most had considerably dilated mitral anulae; the other 6 had ruptured chordae tendineae with less degrees of anular dilatation. Of the 17 patients without morphologic MVP, 3 had echo MVP (coronary artery disease in 1 and infective endocarditis on a previous normal valve in 2); of the 14 with neither echo nor morphologic MVP, 9 had papillary muscle dysfunction from coronary artery disease, 4 had infective endocarditis on previously normal valves and 1 had rheumatic heart disease. The patients with very dilated mitral anuli and leaflet areas generally had holosystolic (hammocking) patterns on echo; the patients with small anuli and leaflet areas usually had mid-to-late systolic (buckling) prolapse patterns.

Publication:

Waller, B. F., Maron, B. J., Del Negro, A. A., Gottdiener, J. S., Roberts, W. C.: Frequency and Significance of M-Mode Echocardiographic Evidence of Mitral Valve Prolapse in Clinically Isolated Pure Mitral Regurgitation: Analysis of 65 Patients Having Mitral Valve Replacement. The American Journal of Cardiology 53:139-147, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03800-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Amounts of Coronary Arterial Narrowing by Atherosclerotic Plaques in Clinically

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Paul J. Day, Student, Pathology Branch, NHLBI
Bruce M. McManus, Staff Fellow, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS.	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The degree of cross-sectional area (XSA) narrowing by atherosclerotic plaque in each of the 4 major epicardial coronary arteries (right, left main, left anterior descending and left circumflex) was determined at necropsy in 37 patients (30 men and 7 women) aged 34 to 77 years (mean 54) with severe, isolated, chronic, pure aortic regurgitation (AR). In 7 patients (19%), $\geq$ 1 major coronary artery was narrowed 76 to 100% in XSA at some point. Of the 148 major coronary arteries examined in the 37 patients, 12 arteries (8%) were narrowed at some point 76 to 100% in XSA. Each of the 148 major coronary arteries were divided into 5-mm-long segments (average 53 per patient) and a histologic section from each segment was examined. Of the 1,977 segments, 1,087 were narrowed 0 to 25%, 669 (34%) 26 to 50%, 170 (9%) 51 to 75%, 48 (2%) 76 to 95% and 3 (0.001%) 96 to 100%. The average amount of XSA narrowing by atherosclerotic plaque per segment was about 28%. Of the 37 patients, 9 had had angina pectoris, 2 of whom had significant (>75% XSA reduction) coronary narrowing; 2 other patients had had acute myocardial infarction clinically, 1 of whom had significant coronary narrowing at necropsy. Thus, in general, the amount of coronary narrowing in our 37 adults with severe, pure, isolated, chronic AR was realtively mild.

200

Project Description:

The degree of cross-sectional area (XSA) narrowing by atherosclerotic plaque in each of the 4 major epicardial coronary arteries (right, left main, left anterior descending and left circumflex) was determined at necropsy in 37 patients (30 men and 7 women) aged 34 to 77 years (mean 54) with severe, isolated, chronic, pure aortic regurgitation (AR). In 7 patients (19%), $\geq$ 1 major coronary artery was narrowed 76 to 100% in XSA at some point. Of the 148 major coronary arteries examined in the 37 patients, 12 arteries (8%) were narrowed at some point 76 to 100% in XSA. Each of the 148 major coronary arteries were divided into 5-mm-long segments (average 53 per patient) and a histologic section from each segment was examined. Of the 1,977 segments, 1,087 were narrowed 0 to 25%, 669 (34%) 26 to 50%, 170 (9%) 51 to 75%, 48 (2%) 76 to 95% and 3 (0.001%) 96 to 100%. The average amount of XSA narrowing by atherosclerotic plaque per segment was about 28%. Of the 37 patients, 9 had had angina pectoris, 2 of whom had significant (>75% XSA reduction) coronary narrowing; 2 other patients had had acute myocardial infarction clinically, 1 of whom had significant coronary narrowing at necropsy. Thus, in general, the amount of coronary narrowing in our 37 adults with severe, pure, isolated, chronic AR was realtively mild.

Publication:

Day, P. J., McManus, B. M. and Roberts, W. C." Amounts of Coronary Arterial Narrowing by Atherosclerotic Plaques in Clinically Isolated, Chronic, Pure Aortic Regurgitation: Analysis of 37 Necropsy Patients Older than 30 Years. The American Journal of Cardiology 53:173-177, 1984.

801

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03801-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Relation of Level of Total Serum Cholesterol to Amount of Calcific Deposits in

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Paul J. Day, Student, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This report analyzes 155 patients with rheumatic mitral stenosis in whom the operatively excised mitral valve was x-rayed to determine the presence of an extent of calcific deposits and the preoperative level of total serum cholesterol (TC). The amount of mitral calcium was graded 0 to 4+ and the average TC for each of the 5 groups was: 0 deposits - 21 patients (14%) (TC = 188 mg/dl); 1+ - 50 patients (32%) (TC = 196 mg/dl); 2+ - 22 patients (14%) (TC = 198 mg/dl); 3+ - 37 patients (24%) (TC = 205 mg/dl), and 4+ - 25 patients (16%) (TC = 184 mg/dl). These average values of TC and the mean ages of the patients in each of the 5 groups of mitral calcium were not significantly different.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

This report analyzes 155 patients with rheumatic mitral stenosis in whom the operatively excised mitral valve was x-rayed to determine the presence of an extent of calcific deposits and the preoperative level of total serum cholesterol (TC). The amount of mitral calcium was graded 0 to 4+ and the average TC for each of the 5 groups was: 0 deposits - 21 patients (14%) (TC = 188 mg/dl); 1+ - 50 patients (32%) (TC = 196 mg/dl); 2+ - 22 patients (14%) (TC = 198 mg/dl); 3+ - 37 patients (24%) (TC = 205 mg/dl), and 4+ - 25 patients (16%) (TC = 184 mg/dl). These average values of TC and the mean ages of the patients in each of the 5 groups of mitral calcium were not significantly different.

Publication:

Day, P. J. and Roberts, W. C.: Relation of Level of Total Serum Cholesterol to Amount of Calcific Deposits in Operatively Excised Stenotic Mitral Valves: Analysis of 155 Cases. The American Journal of Cardiology 53:157-159, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03802-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

A Precaution When Using the St. Jude Medical Prosthesis in the Aortic Valve Position

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Elizabeth M. Ross, Staff Fellow, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The St. Jude Medical (SJM) prosthesis is increasingly being used for cardiac valve replacement. In 1980, the manufacturer supplied 9,000 SJM prostheses to surgeons, and in 1983 this number had risen to 18,500. From 1977 through 1983, 45,000 SJM prostheses were supplied to surgeons. The SJM prosthesis appears to be durable, its orifice area is larger for its size than any other mechanical prosthesis or for any tissue valve mounted on a frame, its low profile prevents interference to leaflet movement by adjacent tissues, and it appears to be less destructive to blood elements than any other mechanical prosthesis. Its major disadvantage is its delicate hinge mechanism which potentially could be interferred with more easily than other mechanical prostheses by thrombus or suture or a calcific deposit. Additionally, like all mechanical prosthetic valves, anticoagulants are required. The SJM prosthesis is a bit more expensive than other presently available mechanical prostheses or bioprostheses.

Recently, we examined the hearts of 2 patients who died early after replacement of the aortic valve with a SJM prosthesis and in each it appeared that movement of 1 of the 2 SJM prosthetic leaflets was interferred with by underlying severe hypertrophy and leftward positioning of the ventricular septum in 1 patient (who died 6 days after aortic valve replacement) or by underlying severely thickened anterior mitral leaflet in the other patient (who could not be separated from cardiopulmonary bypass).

The most likely circumstance in which the ventricular septum could prevent the application of adequate pressure being applied to the undersurface of a SJM leaflet in the aortic valve position is in the older patient with aortic valve stenosis.

804

Project Description:

The St. Jude Medical (SJM) prosthesis is increasingly being used for cardiac valve replacement. In 1980, the manufacturer supplied 9,000 SJM prostheses to surgeons, and in 1983 this number had risen to 18,500. From 1977 through 1983, 45,000 SJM prostheses were supplied to surgeons. The SJM prosthesis appears to be durable, its orifice area is larger for its size than any other mechanical prosthesis or for any tissue valve mounted on a frame, its low profile prevents interference to leaflet movement by adjacent tissues, and it appears to be less destructive to blood elements than any other mechanical prosthesis. Its major disadvantage is its delicate hinge mechanism which potentially could be interferred with more easily than other mechanical prostheses by thrombus or suture or a calcific deposit. Additionally, like all mechanical prosthetic valves, anticoagulants are required. The SJM prosthesis is a bit more expensive than other presently available mechanical prostheses or bioprostheses.

Recently, we examined the hearts of 2 patients who died early after replacement of the aortic valve with a SJM prosthesis and in each it appeared that movement of 1 of the 2 SJM prosthetic leaflets was interferred with by underlying severe hypertrophy and leftward positioning of the ventricular septum in 1 patient (who died 6 days after aortic valve replacement) or by underlying severely thickened anterior mitral leaflet in the other patient (who could not be separated from cardiopulmonary bypass).

The most likely circumstance in which the ventricular septum could prevent the application of adequate pressure being applied to the undersurface of a SJM leaflet in the aortic valve position is in the older patient with aortic valve stenosis.

Publication:

Ross, E. M., and Roberts, W. C.: A Precaution When Using The St. Jude Medical Prosthesis in the Aortic Valve Position. American Journal of Cardiology 54:231-233, 1984.

805

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03803-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders)*

Late (5-132 months) Clinical and Hemodynamic Results AFter Either Tricuspid Valve

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Marc A. Silver, Medical Staff Fellow, Pathology Branch, NHLBI
Steven R. Cohen, Surgery Branch, NHLBI
Charles L. McIntosh, Senior Attending Surgeon, Surgery Branch, NHLBI
Richard O. Cannon, Cardiology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Surgery Branch and Cardiology Branch, NHLBI

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use stendard unreduced type Do not exceed the space provided)*

Late clinical and hemodynamic observations are described in 6 patients who had either tricuspid valve anuloplasty (TVA) (2 patients) or tricuspid valve replacement (TVR) (4 patients) 5 to 132 months earlier for Ebstein's anomaly of the tricuspid valve unassociated with right ventricular outflow obstruction. Of the 6 patients, 5 postoperatively had improved by 1 New York Heart Association functional class and 1, by 2 functional classes. The cardiothoracic ratio decreased 5-12 months after either TVR or TVA in all 6 patients (from a mean of 0.72 to 0.62). Repeat cardiac catheterization 5-12 months after TVA or TVR disclosed that the right atrial mean pressure had increased from a median of 4.0 to 10.5 mm Hg ($p=0.05$); that the right ventricular peak systolic pressure had increased from 19.0 to 31.5 mm Hg ($p=0.02$); that the right ventricular end-diastolic pressure had increased from 5.0 to 9.0 mm Hg ($p=0.05$); that the systemic arterial peak systolic pressure had increased from 115 to 123 mm Hg ($p=0.03$), and that the cardiac index had increased (in all 4 patients in whom both pre- and postoperative values were available) from 1.7 to 2.9 $L/min/m^2$ ($p=0.06$). Thus, the tricuspid valve operations in our 6 patients with Ebstein's anomaly were associated with a decrease in symptoms of cardiac dysfunction, a decrease in cardiac size, an increase in cardiac index and an increase in right ventricular and right atrial pressures. The elevation of the right atrial pressures postoperatively may have resulted from increased right ventricular filling pressures, persistent tricuspid regurgitation, or bioprosthetic stenosis.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Late clinical and hemodynamic observations are described in 6 patients who had either tricuspid valve anuloplasty (TVA) (2 patients) or tricuspid valve replacement (TVR) (4 patients) 5 to 132 months earlier for Ebstein's anomaly of the tricuspid valve unassociated with right ventricular outflow obstruction. Of the 6 patients, 5 postoperatively had improved by 1 New York Heart Association functional class and 1, by 2 functional classes. The cardiothoracic ratio decreased 5-12 months after either TVR or TVA in all 6 patients (from a mean of 0.72 to 0.62). Repeat cardiac catheterization 5-12 months after TVA or TVR disclosed that the right atrial mean pressure had increased from a median of 4.0 to 10.5 mm Hg ($p=0.05$); that the right ventricular peak systolic pressure had increased from 19.0 to 31.5 mm Hg ($p=0.02$); that the right ventricular end-diastolic pressure had increased from 5.0 to 9.0 mm Hg ($p=0.05$); that the systemic arterial peak systolic pressure had increased from 115 to 123 mm Hg ($p=0.03$), and that the cardiac index had increased (in all 4 patients in whom both pre- and postoperative values were available) from 1.7 to 2.9 $L/min/m^2$ ($p=0.06$). Thus, the tricuspid valve operations in our 6 patients with Ebstein's anomaly were associated with a decrease in symptoms of cardiac dysfunction, a decrease in cardiac size, an increase in cardiac index and an increase in right ventricular and right atrial pressures. The elevation of the right atrial pressures postoperatively may have resulted from increased right ventricular filling pressures, persistent tricuspid regurgitation, or bioprosthetic stenosis.

Publication: Silver, M. A., Cohen, S. R., McIntosh, C. L., Cannon, R. O., and Roberts, W. C.:
Late (5-132 months) Clinical and Hemodynamic Results After Either Tricuspid Valve Replacement or Anuloplasty for Ebstein's Anomaly of the Tricuspid Valve. American Journal of Cardiology 54:627-632, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03804-01 PA

PERIOD COVERED
October 1, 1983 to September 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
The Heart in Massive (>300 pounds or >136 kilograms) Obesity: Analysis of 12

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Observations are described in 12 massively (>300 pounds) obese patients aged 25 to 59 years (mean 37) (5 women) weighing 312 to >500 pounds (mean 381): 7 had had systemic hypertension; 4, hypersomnia or sleep apnea; 2, diabetes mellitus, and 1, symptomatic coronary artery disease. Five died suddenly from undetermined causes; 2, from right-sided congestive heart failure; 1, from acute myocardial infarction; 1, from aortic dissection; 1, from intracerebral hemorrhage; 1, from a drug overdose, and 1, post-operatively shortly after an ileal bypass. The heart weight was increased in all 12 patients. The heart weight to body weight ratio expressed as a percent ranged from 0.22 to 0.61 (mean 0.37) (normal for men = 0.42 to 0.46 [mean 0.43]; for women = 0.38 to 0.46 [mean 0.40]). The left ventricular cavity was dilated in 11 patients, and the right ventricular cavity, in 12. Only 2 patients (aged 42 and 59 years) had 1 or more major epicardial coronary arteries narrowed >75% in cross-sectional area (XSA) by atherosclerotic plaque, 1 of whom had no symptoms of myocardial ischemia. Of 664 five-mm segments from the 4 major epicardial coronary arteries from 11 patients (mean 60 per patient), 431 (65%) were narrowed 0-25% in XSA; 143 (21%), 26-50%; 73 (11%), 51-75%, and 17 (3%), 76-100%. Thus, these extremely obese patients who died prematurely did not have more coronary atherosclerosis than might be expected for their ages.

808

Project Description:

Observations are described in 12 massively (>300 pounds) obese patients aged 25 to 59 years (mean 37) (5 women) weighing 312 to >500 pounds (mean 381): 7 had had systemic hypertension; 4, hypersomnia or sleep apnea; 2, diabetes mellitus, and 1, symptomatic coronary artery disease. Five died suddenly from undetermined causes; 2, from right-sided congestive heart failure; 1, from acute myocardial infarction; 1, from aortic dissection; 1, from intracerebral hemorrhage; 1, from a drug overdose, and 1, post-operatively shortly after an ileal bypass. The heart weight was increased in all 12 patients. The heart weight to body weight ratio expressed as a percent ranged from 0.22 to 0.61 (mean 0.37) (normal for men = 0.42 to 0.46 [mean 0.43]; for women = 0.38 to 0.46 [mean 0.40]). The left ventricular cavity was dilated in 11 patients, and the right ventricular cavity, in 12. Only 2 patients (aged 42 and 59 years) had 1 or more major epicardial coronary arteries narrowed >75% in cross-sectional area (XSA) by atherosclerotic plaque, 1 of whom had no symptoms of myocardial ischemia. Of 664 five-mm segments from the 4 major epicardial coronary arteries from 11 patients (mean 60 per patient), 431 (65%) were narrowed 0-25% in XSA; 143 (21%), 26-50%; 73 (11%), 51-75%, and 17 (3%), 76-100%. Thus, these extremely obese patients who died prematurely did not have more coronary atherosclerosis than might be expected for their ages.

Publication:

Warnes, C. A., and Roberts, W. C.: The Heart in Massive (>300 pounds or >136 kilograms) Obesity: Analysis of 12 Patients Studied At Necropsy. American Journal of Cardiology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03805-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chererters or less Title must fit on one line between the borders.)*
Anorexia Nervosa, Sudden Death and Q-T Interval Prolongation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Jeffrey M. Isner, Departments of Medicine (Cardiology) and Pathology, Tufts-New England Medical Center, Boston, MA
William C. Roberts, Chief, Pathology Branch, NHLBI
Steven B. Heymsfield, Division of Nutrition, Emory University Hospital, Atlanta,Ga
Joel Yager, Neuropsychiatric Institute, University of California at Los Angeles,CA

COOPERATING UNITS *(if any)*
Tufts-New England Medical Center, Boston, MA; Emory University Hospital, Atlanta, GA; Neuropsychiatric Institute, University of California at Los Angeles, CA

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Because QT-interval prolongation (QTIP) has been associated with sudden death inpatients using liquid protein diets, the role of QTIP was investigated in 3 women, aged 29, 32 and 37 years, with anorexia nervosa in whom both necropsy and electrocardiograms were available for review. At death, they weighed 41.8, 23.5, and 31.0 kg, respectively. Electrocardiogram recorded < 7 days before death in each shoed prolonged Q-T intervals: 0.61, 0.47, and 0.46 sec, respectively, when corrected for heart rate. Terminal ventricular tachyarrhythmias were documented in 2 patients, including torsade de pointes in 1. The clinical and necropsy findings in these 3 patients provide evidence that sudden death in anorexia nervosa may result from QTIP and indicate the need for electrocardiographic monitorin of such patients.

810

Project Description:

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Publications:

Isner, J. M., Roberts, W. C., Heymsfield, S. B. and Yager, J.: Anorexia Nervosa, Sudden Death and Q-T Interval Prolongation. Annals of Internal Medicine, in press.

811

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03806-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Cardiac Involvement by Kaposi's Sarcoma in Acquired Immune Deficiency Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marc A. Silver, Medical Staff Fellow, Pathology Branch, NHLBI
Abe M. Macher, Laboratory of Pathology, National Cancer Institute
Cheryl M. Reichert
David L. Levens
Joseph E. Parrillo
Dan L. Longo
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Pathology, and Medine Branch, National Cancer Institute, and Critical Care Medicine Department, The Clinical Center, NIH

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Acquired immune deficiency syndrome (AIDS), a recently described disease affecting primarily homosexuals and intravenous drug abusers, has generated considerable interest. At the National Institutes of Health we have performed autopsies on 18 patients with AIDS. General necropsy findings in the first 10 patients recently was reported. This report focuses specifically on cardiac findings in the 18 patients.

Of the 18 patients, 5 (28%) had abnormalities in the heart and in each they consisted of focal deposits of Kaposi's sarcoma (KS). All 5 patients were white men aged 26-52 years (mean 41). The initial manifestation of AIDS in each was a dermal lesion which on biopsy was KS. The interval from the appearance of the dermal KS to death ranged from 6-22 months (mean 11). During life, no patient had symptoms attributable to cardiac dysfunction. Electrocardiograms recorded during the last 3.5 months of life were normal in all 5 patients. All 5 had normal sized hearts by chest roentgenogram and each had normal sized hearts at necropsy.

At necropsy, small, focal, hemorrhagic areas involved subepicardial adipose tissue adjacent to >1 major coronary arteries in all 5 patients. The KS was similar histologically to that seen in other anatomic sites, and was typical of what was has been previously described. Three patients also had deposits of KS in the adventitia of ascending aorta, and 1 of them also had KS in the adventitia of the pulmonary trunk. The myocardium and endocardium was free of KS. The lumens of the coronary arteries were normal in 4 patients, and 1 major coronary artery, not involved by KS, was narrowed 51-75% in cross-sectional area by atherosclerotic plaque in 1 patient. No infectious complications of AIDS were identified histologically. Toxoplasma gondii, however, was isolated in 1 patient from mice injected intraperitoneally with a homogenate of myocardium.

8/2

Project Description:

Acquired immune deficiency syndrome (AIDS), a recently described disease affecting primarily homosexuals and intravenous drug abusers, has generated considerable interest. At the National Institutes of Health we have performed autopsies on 18 patients with AIDS. General necropsy findings in the first 10 patients recently was reported. This report focuses specifically on cardiac findings in the 18 patients.

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At necropsy, small, focal, hemorrhagic areas involved subepicardial adipose tissue adjacent to $\geq$1 major coronary arteries in all 5 patients. The KS was similar histologically to that seen in other anatomic sites, and was typical of what was has been previously described. Three patients also had deposits of KS in the adventitia of ascending aorta, and 1 of them also had KS in the adventitia of the pulmonary trunk. The myocardium and endocardium was free of KS. The lumens of the coronary arteries were normal in 4 patients, and 1 major coronary artery, not involved by KS, was narrowed 51-75% in cross-sectional area by atherosclerotic plaque in 1 patient. No infectious complications of AIDS were identified histologically. Toxoplasma gondii, however, was isolated in 1 patient from mice injected intraperitoneally with a homogenate of myocardium.

Publication:

Silver, M. A., Macher, A. M., Reichert, C. M., Levens, D. L., Parrillo, J. E., Longo, D. L., and Roberts, W. C.: Cardiac Involvement by Kaposi's Sarcoma in Acquired Immune Deficiency Syndrome (AIDS). American Journal of Cardiology 53:983-985, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03807-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Comparison of 7 English-Language Cardiology Journals for 1983

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This article analyzes and compares 7 English-language cardiology journals for the year 1983 in terms of numbers of pages, articles, types of articles, figures and tables published and numbers of authors per article. The 7 journals included the 4 major USA cardilogy journals -- The American Journal of Cardiology (AJC), Circulation, Journal of American College of Cardiology (JACC) and American Heart Journal (AHJ) -- and 3 non-USA English-language cardiology journals: British Heart Journal (BHJ), European Heart Journal (EHJ) and the International Journal of Cardiology (IJC). Although Circulation published the most total pages (because of its huge abstract issue), the AJC published more total pages for articles, the most articles (in its regular issues, 44% more than Circulation, 48% more than the JACC and 39% more than the AHJ) and the AJC provided the most words per page (its maximal number being 20% higher than the maximal number in Circulation, 16% higher than in the JACC and 26% higher than in the AHJ). Each of the 3 non-USA journals was much smaller in terms of pages and articles published than were any of the 4 USA journals. In types of articles published, several differences among the 7 journals were apparent. Of the articles in Circulation, 24% concerned experimental (nonhuman) studies; this percent was 14 in the JACC, 12 in the AHJ, 8 in the AJC and none or nearly none in the BHJ, EHJ and IJC. Brief reports accounted for 27% of the 392 articles in the AHJ and 13% of the 643 articles in the AJC. Of the 190 articles in the IJC, 112 (59%) were editorials and 14% were brief reports (case reports). Articles concerning systemic hypertension accounted for only 2% of the 2,250 articles published in the regular issues of all 7 journals. The 4 USA journals averaged 4.7 authors per article and the 3 non-USA journals, 3.4 authors per article.

814

Project Description:

This article analyzes and compares 7 English-language cardiology journals for the year 1983 in terms of numbers of pages, articles, types of articles, figures and tables published and numbers of authors per article. The 7 journals included the 4 major USA cardilogy journals -- The American Journal of Cardiology (AJC), Circulation, Journal of American College of Cardiology (JACC) and American Heart Journal (AHJ) -- and 3 non-USA English-language cardiology journals: British Heart Journal (BHJ), European Heart Journal (EHJ) and the International Journal of Cardiology (IJC). Although Circulation published the most total pages (because of its huge abstract issue), the AJC published more total pages for articles, the most articles (in its regular issues, 44% more than Circulation, 48% more than the JACC and 39% more than the AHJ) and the AJC provided the most words per page (its maximal number being 20% higher than the maximal number in Circulation, 16% higher than in the JACC and 26% higher than in the AHJ). Each of the 3 non-USA journals was much smaller in terms of pages and articles published than were any of the 4 USA journals. In types of articles published, several differences among the 7 journals were apparent. Of the articles in Circulation, 24% concerned experimental (nonhuman) studies; this percent was 14 in the JACC, 12 in the AHJ, 8 in the AJC and none or nearly none in the BHJ, EHJ and IJC. Brief reports accounted for 27% of the 392 articles in the AHJ and 13% of the 643 articles in the AJC. Of the 190 articles in the IJC, 112 (59%) were editorials and 14% were brief reports (case reports). Articles concerning systemic hypertension accounted for only 2% of the 2,250 articles published in the regular issues of all 7 journals. The 4 USA journals averaged 4.7 authors per article and the 3 non-USA journals, 3.4 authors per article.

Publication:

Roberts, W. C.: Comparison of 7 English-Language Cardiology Journals for 1983. American Journal of Cardiology 53:862-869, 1984.

815

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03808-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Floating Heart or the Heart too Fat to Sink: Analysis of 55 Necropsy Patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
William C. Roberts, Chief, Pathology Branch, NHLBI
John D. Roberts, student volunteer, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Certain clinical and morphologic findings are described in 55 patients whose hearts at necropsy contained so much fat that they floated in water. The patients were 47 to 89 years old (mean 67). Symptomatic coronary heart disease was present in 28 (51%) and valvular heart disease (mitral stenosis) in 3 (5%). The heart at necropsy was enlarged (>350 g for women and >400 g for men) in 45 patients (82%). The mean heart weight for the 31 women was 470 g and for the 14 men, 515 g. In addition to the severe increase in fat in the atrioventricular sulci and over both ventricles, the amount of fat in the atrial septum was increased in all patients. In 14 patients (25%), the thickness of the atrial septum cephaled to the fossa ovale was ≥2 cm. Excessive fat in this location is called "lipomatous hypertrophy of the atrial seputm." Of the 16 patients (29%) with fatal acute myocardial infarction, 7 (44%) had rupture of either the left ventricular free wall or ventricular septum. The high frequency of ardiac rupture in these patients supports the contention that rupture during acute myocardial infarction is more common in the fatty than in the non-fatty heart.

8/6

Project Description:

Certain clinical and morphologic findings are described in 55 patients whose hearts at necropsy contained so much fat that they floated in water. The patients were 47 to 89 years old (mean 67). Symptomatic coronary heart disease was present in 28 (51%) and valvular heart disease (mitral stenosis) in 3 (5%). The heart at necropsy was enlarged (>350 g for women and >400 g for men) in 45 patients (82%). The mean heart weight for the 31 women was 470 g and for the 14 men, 515 g. In addition to the severe increase in fat in the atrioventricular sulci and over both ventricles, the amount of fat in the atrial septum was increased in all patients. In 14 patients (25%), the thickness of the atrial septum cephaled to the fossa ovale was $\geq$2 cm. Excessive fat in this location is called "lipomatous hypertrophy of the atrial seputm." Of the 16 patients (29%) with fatal acute myocardial infarction, 7 (44%) had rupture of either the left ventricular free wall or ventricular septum. The high frequency of cardiac rupture in these patients supports the contention that rupture during acute myocardial infarction is more common in the fatty than in the non-fatty heart.

Publication:

Roberts, W. C., and Roberts, J. D.: The Floating Heart or the Heart too Fat to Sink: Analysis of 55 Necropsy Patients. The American Journal of Cardiology 52:1286-1289.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03809-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

~~A Simple Method to Interpret Cardiac and Aortic Anatomy from Chest Radiographs~~

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

T. James Waters, Division of Cardiology, Department of Medicine, Georgetown University Medical Center, Washington, D.C.

Richard E. Rubin, Division of Cardiology, Department of Medicine, Georgetown University Medical Center, Washington, D.C.

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*
Division of Cardiology, Department of Medicine, Georgetown University Medical Center, Washington, D.C.

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- ☒ xx (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This report describes a simple technique to aid in the interpretation of the cardiac and aortic silhouette on chest roentgenogram. A 3 x 5 inch (7.6 x 12.7 mm) card is cut so that it is square (3 x 3 inches) (7.6 x 7.6 mm). On the card, 4 equal-sized circles are drawn as demonstrated. The circle closest to the bottom of the card represents right venticle (RV), the 1 to the right, right atrium (RA), the 1 toward the top of the card, left atrium (LA) and the 1 to the left, left ventricle (LV). Between the RA and RV, of course, is the tricuspid valve (TV), and between the LA and LV, the mitral valve (MV). The aortic valve (AV) lies in the center surrounded by the circles representing each of the 4 cardiac chambers. The descending thoracic aorta (A) is located to the left of the mtiral valve. The esophagus is located between the LA and vertebral bodies.

After these structures are drawn on the 3 x 3 card, the ends of a pipe-cleaning wick are placed through the aortic valve and descending aorta and secured by bending the wire on the back of the card. The wick then represents the ascending, transverse and proximal descending thoracic aorta. The bottom of the card represents the anterior chest wall, the right side of the card, the right side of the chest, the top of the card, the posterior wall, and the left edge of the card, the left lateral wall of the chest. The card can now be viewed from the front, left lateral position, left anterior oblique position and from the right anterior oblique postion, simply by rotating the card.

This simple index (3 x 3 inch) card with the pipe wick can be kept in the wallet or purse until these chamber locations in each of the 4 standard radiographic views are engrained. This simplified approach has made the teaching of radiographic anatomy easy.

818

Project Description:

This report describes a simple technique to aid in the interpretation of the cardiac and aortic silhouette on chest roentgenogram. A 3 x 5 inch (7.6 x 12.7 mm) card is cut so that it is square (3 x 3 inches) (7.6 x 7.6 mm). On the card, 4 equal-sized circles are drawn as demonstrated. The circle closest to the bottom of the card represents right venticle (RV), the 1 to the right, right atrium (RA), the 1 toward the top of the card, left atrium (LA) and the 1 to the left, left ventricle (LV). Between the RA and RV, of course, is the tricuspid valve (TV), and between the LA and LV, the mitral valve (MV). The aortic valve (AV) lies in the center surrounded by the circles representing each of the 4 cardiac chambers. The descending thoracic aorta (A) is located to the left of the mtiral valve. The esophagus is located between the LA and vertebral bodies.

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This simple index (3 x 3 inch) card with the pipe wick can be kept in the wallet or purse until these chamber locations in each of the 4 standard radiographic views are engrained. This simplified approach has made the teaching of radiographic anatomy easy.

Publication:

Waters, T. J., Rubin, R. E., and Roberts, W. C.: A Simple Method to Interpret Cardiac and Aortic Anatomy From Chest Radiographs. American Journal of Cardiology 52:644-645, 1983.

819

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03810-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Coronary Artery Surgery Study (CASS): Do the Results Apply to Your Patient?

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
William C. Roberts, Chief, Pathology Branch, NHLBI
Dennis M. Manning, Division of Cardiology (Fellow), Department of Medicine,
The Mercy Hospital of Pittsburgh, Pittsburgh, PA
(1 month fellowship in Pathology Branch, NHLBI)

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This editorial comments on the usefulness of the CASS study to the physicians seeing patients with cardiac disease. Despite the enormous amount of information thus far provided by the 24 - million dollar CASS, utilization of its results will not be easy. CASS implies that the patient who has mild angina pectoris or who is asymptomatic after healing of AMI does not need CABG (or possibly percutaneous transluminal coronary angioplasty) at least at the present time. Because coronary bypass or dilatation is unnecessary in this asymptomatic or mildly symptomatic state, justification for angiography to determine the status of the coronary arteries and left ventricle is lacking. But, if angiography is not performed, the data acquired in CASS is really not applicable. If angiography is performed in the asymptomatic or mildly symptomatic patient, the finding of $\geq$70% diameter reduction in $\geq$2 major (excluding left main) coronary arteries may result, in probably many medical centers, in the performance of coronary bypass or dilatation. The major worry in avoiding the performance of cardiac catheterization would be the missing of severe ($\geq$70% diameter reduction) narrowing of the left main coronary artery. But, severe left main narrowing, was found in <2% of the more than 16,000 subjects screened for randomization. It therefore would appear most reasonable in light of CASS not to perform coronary angiography in the patient who has mild angina (with or without previous AMI which healed) or is asymptomatic after healing of AMI. When symptoms of myocardial ischemia appear or worsen, coronary (and left ventricular) angiography can then be performed with coronary dilatation and/or bypass thereafter if appropriate. A major implication of CASS, therefore, is to avoid the performance of coronary (and left ventricular) angiography until symptoms of myocardial ischemia become moderate or severe (as opposed to absent or mild) despite medical therapy.

$20

Project Description:

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Publication:

Roberts, W. C. and Manning, D. M.: The Coronary Artery Surgery Study (CASS): Do the results apply to your patient? American Journal of Cardiology 54:440-443, 1984.

f21

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03811-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Aortic Valve Stenosis: Estimating the Severity of Obstruction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI
Robert J. Siegel, Staff Fellow, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Subtraction of the indirect systemic arterial systolic pressure, (in mm Hg) form the total 12-lead QRS amplitude (in mm) may provide a reasonable noninvasive prediction of the peak systolic pressure gradient across the aortic valve in patients with moderate to severe aortic valve stenosis (AS). Most studies of the electrocardiogram (ECG) in patients with AS have involved living patients in whom the status of the left ventricular myocardium, epicardial coronary arteries, and mitral valve was not precisely known. We examined the 12-lead ECG recorded within 2 months of death in 50 patients aged 16-65 years (mean age: 48) with peak systolic pressure gradients across the aortic valve in the range of 52-180 mm Hg (mean: 98) and anatomically normal mitral valves. Excluding 4 patients with complete left bundle branch block (LBBB), 44 (96 per cent) of the other 46 patients had the usual voltage criteria for left ventricular hypertrophy. Measurement of the total 12-lead QRS amplitude, which ranged from 144 to 417 mm (10 mm = 1mV; mean : 257) correlated directly with the peak systolic pressure gradient across the aortic valve and, when the 4 patients with complete LBBB were excluded, with the peak left ventricular systolic pressure. The total 12-lead QRS amplitude (in mm) was similar in most patients to the left ventricular systolic pressure (in mm Hg). The mean of the total 12-lead QRS amplitude was significantly ($P<0.05$) greater in the 11 younger (<40 years) than in the 39 older patients (278 mm vs 257 mm), in the 14 women than in the 36 men (277 mm vs 249 mm), in the 22 patients with heavier (>600 g) hearts (274 mm vs 244 mm), in the 34 patients without, compared to the 16 with, significant coronary arterial narrowing (270 mm vs 238 mm), and in the 22 patients without, compared to the 24 with, myocardial damage patterns on ECG: 269 mm vs 236 mm.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

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Publication:

Roberts, W. C. and Siegel, R. J.: Aortic Valve Stenosis: Estimating the Severity of Obstruction. Primary Cardiology 10:47-61, 1984.

f23

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03812-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cardiac Valvular Residua and Sequelae After Operation For Congenital Heart Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

This article focuses on valvular abnormalities which remain after operations for congenital anomalies of the heart and great arteries (residua) and on changes occurring later in congenitally malformed cardiac valves subjected to direct operative manipulation to relieve stenosis and/or regurgitation (sequelae).

824

Project Description:

This article focuses on valvular abnormalities which remain after operations for congenital anomalies of the heart and great arteries (residua) and on changes occurring later in congenitally malformed cardiac valves subjected to direct operative manipulation to relieve stenosis and/or regurgitation (sequelae).

Publication:

Roberts, W. C.: Cardiac Valvular Residua and Sequelae After Operation For Congenital Heart Disease. American Heart Journal 106:1181-1187, 1983.

f 25

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03813-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pulmonary Arteries in Congenital Heart Disease: A Structure-Function Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Renu Virmani, Vanderbilt University, Nashville, TN

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our 3-grade anatomic classification of pulmonary arterial changes for patients with pulmonary arterial hypertension is a modification of the classification of Heath and Edwards. Our new classification consists of medial thickening only, grade I; medial and intimal thickening, grade II; and both medial and intimal thickening plus plexiform lesions, grade III. These anatomic changes in the pulmonary arteries result entirely from pulmonary hypertension. Intimal thickening without medial thickening, however, is observed in older persons, and, in itself, is not a consequence of pulmonary hypertension.

Observations in our 87 necropsy patients over age 15 with major congenital cardiovascular malformations indicate that the changes in the pulmonary arteries are directly related to the level of pulmonary arterial pressure. Direction of the shunt, location of the shunt, and age of the patient were important only by their effect on the pulmonary arterial pressure. The patients with PDA had the most severe changes in the pulmonary arteries and the highest average pulmonary arterial pressures.

In patients with pulmonary hypotension (isolated PS) and in patients with PS and VSD)TF), the media of the pulmonary arteries is thinner than normal and the lumens are larger than normal. Intimal thickening is usually seen in patients with TF even though the media is not thickened.

Surgical creation of systemic-to-pulmonary arterial anastomoses may cause the pulmonary arterial media to thicken to normal widths or even to widths thicker than normal, but the development of pulmonary hypertension after such operations is infrequent.

f26

Project Description:

Our 3-grade anatomic classification of pulmonary arterial changes for patients with pulmonary arterial hypertension is a modification of the classification of Heath and Edwards. Our new classification consists of medial thickening only, grade I; medial and intimal thickening, grade II; and both medial and intimal thickening plus plexiform lesions, grade III. These anatomic changes in the pulmonary arteries result entirely from pulmonary hypertension. Intimal thickening without medial thickening, however, is observed in older persons, and, in itself, is not a consequence of pulmonary hypertension.

Observations in our 87 necropsy patients over age 15 with major congenital cardiovascular malformations indicate that the changes in the pulmonary arteries are directly related to the level of pulmonary arterial pressure. Direction of the shunt, location of the shunt, and age of the patient were important only by their effect on the pulmonary arterial pressure. The patients with PDA had the most severe changes in the pulmonary arteries and the highest average pulmonary arterial pressures.

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Surgical creation of systemic-to-pulmonary arterial anastomoses may cause the pulmonary arterial media to thicken to normal widths or even to widths thicker than normal, but the development of pulmonary hypertension after such operations is infrequent.

Publication:

Virmani, R, and Roberts WC: Pulmonary Arteries in Congenital Heart Disease: A Structure-Function Analysis. American Journal of Cardiology in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03814-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Terminology for Location of Acute Myocardial Infarcts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

Julius M. Gardin, Division of Cardiology, Department of Medicine, University of California at Irvine.

COOPERATING UNITS *(if any)*

University of California at Irvine, Irvine, CA

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

The same terminology applicable for describing the location of an AMI at necropsy is applicable for defining its location by electrocardiogram. Certain terms used electrocardiographically, namely "inferior", "diaphragmatic," and "true posterior," should be avoided because their opposites are not used. Ideally, a proper description of the location of an AMI should include defining its involvement in all the dimensions of the left ventricle (considered as a cone): the portion of the walls of the circle involved (anterior, poterior, lateral, and septal); the amount of the wall's thickness involved (transmural or nontransmural [subendocardial]), and the portions of the wall's length involved (basal half or apical half or both). Certain portions of the walls of both the left and right ventricles are rare sites of AMI, and knowing these sites helps in more precisely defining by ECG the location of the AMI. AMI involving the anterior wall of the left ventricle rarely is limited to just its basal half; and, therefore, anterior AMI, for practical purposes, indicates involvement of at least the apical half of the ventricle. AMI involving the basal half of the posterior left ventricular wall, in contrast, is common, but the ECG is not accurate in differentiating posterobasal from posteroapical AMI. Furthermore, the ECG provides no specific pattern to indicate AMI of the ventricular septum, lateral wall of the left ventricle, either posterior or anterolateral walls of the right ventricle, or papillary muscle. AMI of the right ventricle virtually never occurs with "anterior" AMI of the left ventricle. In contrast, nearly 25% of patients with "posterior" transmural AMI also have associated AMI involving at least the posterior wall of the right ventricle.

528

Project Description:

The same terminology applicable for describing the location of an AMI at necropsy is applicable for defining its location by electrocardiogram. Certain terms used electrocardiographically, namely "inferior", "diaphragmatic," and "true posterior," should be avoided because their opposites are not used. Ideally, a proper description of the location of an AMI should include defining its involvement in all the dimensions of the left ventricle (considered as a cone): the portion of the walls of the circle involved (anterior, poterior, lateral, and septal); the amount of the wall's thickness involved (transmural or nontransmural [subendocardial]), and the portions of the wall's length involved (basal half or apical half or both). Certain portions of the walls of both the left and right ventricles are rare sites of AMI, and knowing these sites helps in more precisely defining by ECG the location of the AMI. AMI involving the anterior wall of the left ventricle rarely is limited to just its basal half; and, therefore, anterior AMI, for practical purposes, indicates involvement of at least the apical half of the ventricle. AMI involving the basal half of the posterior left ventricular wall, in contrast, is common, but the ECG is not accurate in differentiating posterobasal from posteroapical AMI. Furthermore, the ECG provides no specific pattern to indicate AMI of the ventricular septum, lateral wall of the left ventricle, either posterior or anterolateral walls of the right ventricle, or papillary muscle. AMI of the right ventricle virtually never occurs with "anterior" AMI of the left ventricle. In contrast, nearly 25% of patients with "posterior" transmural AMI also have associated AMI involving at least the posterior wall of the right ventricle.

Publication:

Roberts, W. C. and Gardin J. M.: Terminology for Location of Acute Myocardial Infarcts. Chapter for book - Acute Myocardial Ischemia and Infarction, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03815-01 PA

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cardiac Morphologic Findings Late After Partial Left Ventricular Endomyocardial

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marc A. Silver, Medical Staff Fellow, Pathology Branch, NHLBI
Andrew I. Cohen, Georgetown University Hospital, Washington, D.C.
Nevin M. Katz, Georgetown Hospital, Washington, D.C.
Ross D. Fletcher, Veterans Administration Hospital, Washington, D.C.
Victor J. Ferrans, Chief, Ultrastructe Section, Pathology Branch, NHLBI
William C.Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Pathology Branch, NHLBI

SECTION:

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Operative excision of portions of left ventricular (LV) endocardium and underlying myocardium often abolishes chronic recurrent sustained ventricular tachycardia (VT) in patients with healed myocardial infarction (MI) with or without associated LV aneurysm. Fenoglio and associates described morphologic features of operatively excised LV subendocardial regions in 23 patients with recurring VT. No studies have described early or late cardiac findings at necropsy in patients having had focal endomyocardial LV resection for recurrent VT. Such is the purpose of this report.

Focal LV endocardial thickening may occur at sites of previous LV mural thrombus, post-operatively at the prior site of a LV vent or myotomy-myectomy (for hypertrophic cardiomyopathy) or overlying a LV myocardial scar. In our patient, dense fibrous thickening devoid of elastic fibers, was observed in the sites of previous endomyocardial resection. One of us (NMK) also re-examined the LV cavity in another patient 6 weeks following endomyocardial resection and similar white, thickened endocardium at the sites of a previous resection was present.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Operative excision of portions of left ventricular (LV) endocardium and underlying myocardium often abolishes chronic recurrent sustained ventricular tachycardia (VT) in patients with healed myocardial infarction (MI) with or without associated LV aneurysm. Fenoglio and associates described morphologic features of operatively excised LV subendocardial regions in 23 patients with recurring VT. No studies have described early or late cardiac findings at necropsy in patients having had focal endomyocardial LV resection for recurrent VT. Such is the purpose of this report.

Focal LV endocardial thickening may occur at sites of previous LV mural thrombus, post-operatively at the prior site of a LV vent or myotomy-myectomy (for hypertrophic cardiomyopathy) or overlying a LV myocardial scar. In our patient, dense fibrous thickening devoid of elastic fibers, was observed in the sites of previous endomyocardial resection. One of us (NMK) also re-examined the LV cavity in another patient 6 weeks following endomyocardial resection and similar white, thickened endocardium at the sites of a previous resection was present.

Publication:

Silver, M. A., Cohen, A. I., Katz, N. M. , Fletcher, R. D., Ferrans, V. J., and Roberts, W. C.: Cardiac Morphologic Findings Late After Partial Left Ventricular Endomyocardial Resection for Recurrent Ventricular Tachycardia. American Journal of Cardiology 54:233-235, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03816-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Eisenmenger Ventricular Septal Defect with Prolonged Survival

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI
James E. Boger, University of Arkansas Medical Center, Little Rock, Arkansas
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*
Un. Arkansas Medical Center, Little Rock, Arkansas

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Most patients with large ventricular septal defect (VSD) are ded by age 40 years. The average age at death in unoperated patients with isolated, large (≥2 cm) VSD is about 35 years. Thus, survival in our patient to 63 years with a 2.5 cm VSD is extraordinary. Indeed, we found detailed descriptions at necropsy in only 5 patients with isolated, large (≥1.5 cm) VSD surviving >40 years without operation. Their ages ranged from 44-60 years (mean 51). Only 1 had cardiac catheterization. The systemic arterial pressure was provided in only 1 patient and it was elevated (indirect = 180/120 mm Hg) as it was in our patient (indirect 170/120 mm Hg).

f32

Project Description:

Most patients with large ventricular septal defect (VSD) are ded by age 40 years. The average age at death in unoperated patients with isolated, large (≥2 cm) VSD is about 35 years. Thus, survival in our patient to 63 years with a 2.5 cm VSD is extraordinary. Indeed, we found detailed descriptions at necropsy in only 5 patients with isolated, large (≥1.5 cm) VSD surviving >40 years without operation. Their ages ranged from 44-60 years (mean 51). Only 1 had cardiac catheterization. The systemic arterial pressure was provided in only 1 patient and it was elevated (indirect = 180/120 mm Hg) as it was in our patient (indirect 170/120 mm Hg).

Publication:

Warnes, C. A., Boger, J. E., and Roberts, W.C.: Eisenmenger Ventricular Septal Defect with Prolonged Survival. American Journal of Cardiology 54:460-462, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03817-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chereclers or less Title must fit on one line between the borders.)*

Eisenmenger Ductus Arteriosus with Prolonged Survival

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigetor) (Neme, title, laboratory, and institute affiliation)*

Bruce M. McManus, Director, Cardiovascular Registry, University of Nebraska Medical Center, Omaha, NE
Peter F. Hahn, Departments of Pathology and Medicine, Brigham and Women's Hosp., Boston, MA
John A. Smith, Dept. Path. and Med., Brigham and Women's Hosp., Boston, MA
William C. Roberts, Chief, Pathology Branch, NHLBI
James H. Jackson, Dept. Path. and Med., Brigham and Women's Hosp., Boston, MA

COOPERATING UNITS *(if eny)*

Departments of Pathology and Medine, Brigham and Women's Hospital, Boston, MA
Cardiovascular Registry, University of Nebraska Medical Center

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Determinants of the ultimate outcome of unoperated patients with ductus arteriosus (DA) are not clearly understood. Generally, the smaller the DA, the smaller the left-to-right shunt, the lower the pulmonary artery (PA) pressure and PA resistance, and the longer the survival. Not all patients, however, with these favorable characteristics do well, and conversely, not all patients with a longstanding large DA and a large left-to-right shunt do poorly. The latter was the case in the patient described in this report. The patient described lived his entire life with elevated PA pressures as reflected by the fetal pattern of elastic laminae in the pulmonary trunk, and during his last 27 years, by measured near-systemic PA pressures. Reports of 23 unoperated patients over 40 years of age with ductus arteriosus studied at necropsy were also summarized in this report.

834

Project Description:

Determinants of the ultimate outcome of unoperated patients with ductus arteriosus (DA) are not clearly understood. Generally, the smaller the DA, the smaller the left-to-right shunt, the lower the pulmonary artery (PA) pressure and PA resistance, and the longer the survival. Not all patients, however, with these favorable characteristics do well, and conversely, not all patients with a longstanding large DA and a large left-to-right shunt do poorly. The latter was the case in the patient described in this report. The patient described lived his entire life with elevated PA pressures as reflected by the fetal pattern of elastic laminae in the pulmonary trunk, and during his last 27 years, by measured near-systemic PA pressures. Reports of 23 unoperated patients over 40 years of age with ductus arteriosus studied at necropsy were also summarized in this report.

Publication:

McManus, B. M., Hahn, P. F., Smith, J. A., Roberts, W. C., Jackson, J. H.: Eisenmenger Ductus Arteriosus with Prolonged Survival. American Journal of Cardiology 54:462-464, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03818-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chereclers or less Title must fit on one line between the borders.)*

Examining the Heart at Necropsy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator) (Name, title, laboretory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if eny)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type Do not exceed the space provided)*

This article summarizes three principles which should be kept in mind when the heart is examined at necropsy.

Principle #1 is to fix the heart in formalin or in some other preserving and firming medium before opening it. Fixation of the specimen before incision allows retention of the three dimensional configuration of the heart and permits more meaningful comparisons between chamber sizes, wall thickness, valve orifice, etc.

Principle #2 is to x-ray the fixed heart specimen before opening it. Radiographs reduce the three-dimensional intact heart to a two-dimensional structure and provide additional means of visualizing chamber sizes and wall thicknesses.

Principle #3 is that the method chosen to open the heart is determined by the type of cardiac disease that is present or suspected. There is no single way to open the heart, and, in general, hearts with different diseases require different methods of opening.

Opening hearts according to the flow of blood, a common practice, is usually the least desirable method of incising hearts.

PHS 6040 (Rev 1/84)

GPO 904 917

Project Description:

This article summarizes three principles which should be kept in mind when the heart is examined at necropsy.

Principle #1 is to fix the heart in formalin or in some other preserving and firming medium before opening it. Fixation of the specimen before incision allows retention of the three dimensional configuration of the heart and permits more meaningful comparisons between chamber sizes, wall thickness, valve orifice, etc.

Principle #2 is to x-ray the fixed heart specimen before opening it. Radiographs reduce the three-dimensional intact heart to a two-dimensional structure and provide additional means of visualizing chamber sizes and wall thicknesses.

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Opening hearts according to the flow of blood, a common practice, is usually the least desirable method of incising hearts.

Publication:

Roberts, W. C.: Examining the Heart At Necropsy. In: The Heart, Hurst, J. W. (ed.), New York, McGraw Hill, in press.

f 37

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03819-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

"Massive" Calcification of a Right Ventricular Outflow Parietal Pericardial Patch

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Elizabeth M. Ross, Senior Staff Fellow, Pathology Branch, NHLBI
Charles L. McIntosh, Senior Attending Surgeon, Surgery Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Surgery Branch, National Heart, Lung, and Blood Institute

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A variety of materials have been employed for patches to widen obstructed right ventricular (RV) outflow tracts in patients with tetralogy of Fallot. These materials have included Teflon, preclotted Dacron, parietal pericardium, and dura mater. Autologous parietal pericardium has the advantage of being readily available, and does not present the problem of suture-line bleeding, which often occurs with the synthetic patches. Both tissue and synthetic patches utilized in the RV outflow tract may become aneurysmal if the RV peak systolic pressure is not returned to normal or near normal levels after operation. Although the intimal lining tissue of the synthetic patch may calcify, the synthetic material itself does not. In contrast, autologous patch material may calcify. Such was the case in the 16-year-old boy described in this report.

F3d

Project Description:

A variety of materials have been employed for patches to widen obstructed right ventricular (RV) outflow tracts in patients with tetralogy of Fallot. These materials have included Teflon, preclotted Dacron, parietal pericardium, and dura mater. Autologous parietal pericardium has the advantage of being readily available, and does not present the problem of suture-line bleeding, which often occurs with the synthetic patches. Both tissue and synthetic patches utilized in the RV outflow tract may become aneurysmal if the RV peak systolic pressure is not returned to normal or near normal levels after operation. Although the intimal lining tissue of the synthetic patch may calcify, the synthetic material itself does not. In contrast, autologous patch material may calcify. Such was the case in the 16-year-old boy described in this report.

Publication:

Ross, E. M., McIntosh, C. L., and Roberts, W. C.: "Massive" Calcification of a Right Ventricular Outflow Parietal Pericardial Patch in Tetralogy of Fallot. American Journal of Cardiology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03820-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Acquired Left Ventricular Endocardial Constriction From Massive Mural Calcific

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marc A. Silver, Medical Staff Fellow, Pathology Branch, NHLBI
Robert O. Bonow, Cardiology Branch, NHLBI
Stewart M. Deglin, Yale University School of Medicine, New Haven, CT
Barry J. Maron , Cardiology Branch, NHLBI
Richard O. Cannon , Cardiology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch, NHLBI, and Yale University School of Medicine, New Haven, CT

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS.	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Impairment to left ventricular (LV) filling can result from left-sided cardiovascular conditions proximal to the left ventricle (mitral stenosis, cor triatriatum, pulmonary venous obstruction), from LV myocardial conditions (including hypertrophic cardiomyopathy, amyloidosis and hemosiderosis), from pericardial constriction (of several etiologies), and from LV endomyocardial fibrosis with or without eosinophilia. The latter condition, occurring primarily in Africa, causes extensive LV endocardial fibrosis located primarily in the LV inflow tract and is associated with little or no mural endocardial calcific deposits. The common denominator of all these conditions is a normal sized or small LV cavity. This report describes a hitherto unreported cause of impairment to LV filling, namely massive LV endocardial calcific deposits associated with LV hypertrophy of undetermined etiology.

The cause of the LV mural endocardial calcific deposits and the associated LV hypertrophy in the above described patient is uncertain. The lack of blood eosinophilia at any time, the presence of considerable LV hypertrophy, and the presence of the extensive, widely distributed calcific deposits within the LV cavity clearly separates our patient from those having "endomyocardial fibrosis with or without eosinophilia" or "Loffler's fibroplastic parietal endocarditis." Irrespective of its cause, however, the entity, massive LV endocardial calcific deposits, needs to be added to the list of causes producing impairment of LV filling.

840

Project Description:

Impairment to left ventricular (LV) filling can result from left-sided cardiovascular conditions proximal to the left ventricle (mitral stenosis, cor triatriatum, pulmonary venous obstruction), from LV myocardial conditions (including hypertrophic cardiomyopathy, amyloidosis and hemosiderosis), from pericardial constriction (of several etiologies), and from LV endomyocardial fibrosis with or without eosinophilia. The latter condition, occurring primarily in Africa, causes extensive LV endocardial fibrosis located primarily in the LV inflow tract and is associated with little or no mural endocardial calcific deposits. The common denominator of all these conditions is a normal sized or small LV cavity. This report describes a hitherto unreported cause of impairment to LV filling, namely massive LV endocardial calcific deposits associated with LV hypertrophy of undetermined etiology.

The cause of the LV mural endocardial calcific deposits and the associated LV hypertrophy in the above described patient is uncertain. The lack of blood eosinophilia at any time, the presence of considerable LV hypertrophy, and the presence of the extensive, widely distributed calcific deposits within the LV cavity clearly separates our patient from those having "endomyocardial fibrosis with or without eosinophilia" or "Loffler's fibroplastic parietal endocarditis." Irrespective of its cause, however, the entity, massive LV endocardial calcific deposits, needs to be added to the list of causes producing impairment of LV filling.

Publication:

Silver, M. A., Bonow, R. O., Deglin, S. M., Maron, B. J., Cannon, R. O., Roberts, W. C.: Acquired Left Ventricular Endocardial Constriction From Massive Mural Calcific Deposits: A Newly Recognized Cause of Impairment to Left Ventricular Filling. American Journal of Cardiology 53:1468-1470, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03821-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders)*
Calcium as a Risk Factor for Coronary Atherosclerosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

xX ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

Irrespective of the precise mechanism of the vascular calcific deposits, it is clear that chronic hypercalcemia is detrimental to the vascular system and that its effect, at least in adults, is worsened by the presence of hypercholesterolemia, and possibly also by the presence of systemic hypertension. Additional support for the role of hypercalcemia in causing vascular and nonvascular calcific deposits comes from studies of chronic hypervitaminoisis D in infants and children, and from its association with the congenital syndrome of peculiar elfin faces, mental retardation, and supravalvular aortic stenosis. The present study also strongly suggests that chronic hypercalcemia is a "risk factor" to premature coronary atherosclerosis, especially in patients with an elevated serum cholesterol.

842

Project Description:

Irrespective of the precise mechanism of the vascular calcific deposits, it is clear that chronic hypercalcemia is detrimental to the vascular system and that its effect, at least in adults, is worsened by the presence of hypercholesterolemia, and possibly also by the presence of systemic hypertension. Additional support for the role of hypercalcemia in causing vascular and nonvascular calcific deposits comes from studies of chronic hypervitaminoisis D in infants and children, and from its association with the congenital syndrome of peculiar elfin faces, mental retardation, and supravalvular aortic stenosis. The present study also strongly suggests that chronic hypercalcemia is a "risk factor" to premature coronary atherosclerosis, especially in patients with an elevated serum cholesterol.

Publication:

Roberts, W. C.: Calcium as a Risk Factor for Coronary Atherosclerosis. IN: Calcium Antagonists and Cardiovascular Disease, Opie, L. H. (ed.), New York, Raven Press, 1984, pp. 147-149.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03822-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Norman Jefferis Holter and Ambulatory ECG Monitoring

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI
Marc A. Silver, Medical Staff Fellow, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This report provides a brief description of the work of Norman Holter who invented Holter monitoring. Holter died on July 21, 1983, and this report was published.

544

Project Description:

This report provides a brief description of the work of Norman Holter who invented Holter monitoring. Holter died on July 21, 1983, and this report was published.

Publication:

Roberts, W. C. and Silver, M. A.: Norman Jefferis Holter and Ambulatory ECG Monitoring. The American Journal of Cardiology 52:903-906, 1983.

845

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03823-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Histiocytoid Cardiomyopathy: A Cause of Sudden Death in Apparently Healthy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey E. Saffitz, Visiting Fellow, Pathology Branch, NHLBI
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
E. Rene Rodriguez, Ultrastructure Section, Pathology Branch, NHLBI
F. Robert Lewis , National Children's Hospital, Washington, D.C.
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [x] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The report describes a previously healthy appearing and normally developed 19-month old girl with histiocytoid cardiomyopathy. At least 20 necropsy patients with this condition in infancy have been reported previously. The unusual feature of the presently reported patient was sudden death.

846

Project Description:

The report describes a previously healthy appearing and normally developed 19-month old girl with histiocytoid cardiomyopathy. At least 20 necropsy patients with this condition in infancy have been reported previously. The unusual feature of the presently reported patient was sudden death.

Publication:

Saffitz, J. E., Ferrans, V. J., Rodriguez, E. R., Lewis, F. R., Roberts, W. C.: Histiocytoid Cardiomyopathy: A Cause of Sudden Death in Apparently Healthy Infants. The American Journal of Cardiology 52:215-217, 1983

847

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03824-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Atrioventricular Septal Defect (Primum Atrial Septal Defect) with Prolonged

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

Gerald I. Shugoll, Georgetown University Medical Center, Washington, D.C.

Robert B. Wallace, Georgetown University Medical Center, Washington, D.C.

William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Georgetown University Medical Center, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Atrioventricular (AV) septal defect (formerly called AV canal or endocardial cushion defect or primum atrial septal defect [ASD] with cleft mitral valve) may be either complete (single AV valve) or partial (2 well formed AV valves). Patients with complete AV septal defect rarely survive without operation for more than a few years. Patients with partial AV septal defect, however, commonly survive for many years but not as long as patients with secundum ASD. Prolonged survival with partial AV septal defect (primum ASD), however, is unusual, particularly in the presence of severe mitral regurgitation and pulmonary hypertension, but such was the case in a 59-year-old woman described in this report. This manuscript reviewed previously reported patients with partial AV septal defect show survival more than 50 years. This is the first report to described mitral anular calcium in a patient with a primum type ASD, as well as, calcium in the pulmonary trunk. Thus, the patient described was unusual on 7 counts (prolonged survival, severe mitral regurgitation, severe pulmonary hypertension, mitral anular calcium, pulmonary truncal calcium, coronary arterial calcium and severe coronary atherosclerosis necessitating aorto-coronary bypass).

848

Project Description:

Atrioventricular (AV) septal defect (formerly called AV canal or endocardial cushion defect or primum atrial septal defect [ASD] with cleft mitral valve) may be either complete (single AV valve) or partial (2 well formed AV valves). Patients with complete AV septal defect rarely survive without operation for more than a few years. Patients with partial AV septal defect, however, commonly survive for many years but not as long as patients with secundum ASD. Prolonged survival with partial AV septal defect (primum ASD), however, is unusual, particularly in the presence of severe mitral regurgitation and pulmonary hypertension, but such was the case in a 59-year-old woman described in this report. This manuscript reviewed previously reported patients with partial AV septal defect show survival more than 50 years. This is the first report to described mitral anular calcium in a patient with a primum type ASD, as well as, calcium in the pulmonary trunk. Thus, the patient described was unusual on 7 counts (prolonged survival, severe mitral regurgitation, severe pulmonary hypertension, mitral anular calcium, pulmonary truncal calcium, coronary arterial calcium and severe coronary atherosclerosis necessitating aorto-coronary bypass).

Publications:

Warnes, C. A., Shugoll, G. I., Wallace, R. B., Roberts, W. C.: Atrioventricular Septal Defect (Primum Strial Septal Defect) with Prolonged Survival (Despite Severe Mitral Regurgitation and Pulmonary Hypertension) and Associated Cardiac Calcification (Mitral Anulus, Coronary Artery and Pulmonary Trunk). The American Journal of Cardiology, in press.

849

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03825-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Diagnosis of Pulmonary Histiocytosis X by Immunodetection of Langerhans Cells.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Françoise Basset, M.D., INSERM U-82, Faculté Xavier Bichat, Paris, France
Sylvie Chollet, Ph.D., INSERM U-82, Faculté Xavier Bichat, Paris, France
Paul Soler, Ph.D., INSERM U-82, Faculté Xavier Bichat, Paris, France
Pierre Dournovo, M.D., INSERM U-82, Faculté Xavier Bichat, Paris, France
Marie S. Richard, M.D., INSERM U-82, Faculté Xavier Bichat, Paris, France

COOPERATING UNITS *(if any)*

INSERM U-82, Faculté Xavier Bichat, Paris, France

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

OKT6 monoclonal antibody was used to detect Langerhans cells in bronchoalveolar lavage fluid from 131 patients in order to study the specificity of the finding of OKT6-positive cells for the diagnosis of pulmonary histiocytosis X. OKT6-reactive cells were found in all patients with histiocytosis X (mean ± SEM, 5.29% ± 1.4% of all cells in lavage fluid from 18 patients). The number of OKT6-positive cells in the other 113 patients was significantly smaller (mean ± SEM, 0.20 ± 0.04% of all cells, $p < 0.001$). However, some overlap was present in a small group of 6 patients who had miscellaneous lung disorders other than pulmonary histiocytosis X and had a positive OKT6 reaction in 1.3 to 2.8% of all cells in lavage fluid. Thus, the finding of OKT6-positive cells in lavage fluid is not absolutely diagnostic of pulmonary histiocytosis X.

$50

Project Description:

Objectives: To study the specificity of the finding of OKT6-positive cells in bronchoalveolar lavage fluid for the diagnosis of pulmonary histiocytosis X.

Methods employed and major findings: Based on the finding that Langerhans cells react with the monoclonal antibody OKT6, raised against a subset of thymocytes, we used this antibody to study the cells collected by bronchoalveolar lavage (BAL) from 131 patients, including 18 with pulmonary histiocytosis X, 43 with pulmonary sarcoidosis, 67 with miscellaneous pulmonary disorders, and 3 controls. Immunofluorescence studies demonstrated the presence of OKT6-reactive cells in all patients with pulmonary histiocytosis X (mean ± SEM, 5.29% ± 1.14% of all cells in BAL fluid). Immunoelectron microscopic studies revealed that the cells labeled in these patients (n = 13) contained Langerhans granules. The number of fluorescent cells in the other 113 patients was significantly smaller (mean ± SEM, 0.20% ± 0.04% of all cells; $P < 0.001$). In the 3 control patients, in the 43 patients with sarcoidosis, and in 61 of the 67 patients with miscellaneous disorders unrelated to histiocytosis X, no cells or < 1% of the total were labeled; however, in the 6 remaining patients in this miscellaneous group, 1.3 to 2.8% of all cells in BAL were labeled. In 3 of these 6 patients, immunoelectron-microscopic examination showed that the cells labeled by OKT6 had the general characteristic of Langerhans cells but lacked Langerhans granules. OKT3, OKT4, and OKT8 monoclonal antibodies did not stain histiocytosis X cells in BAL fluid.

Significance: OKT6 staining is useful for the diagnosis of pulmonary histiocytosis X; however, the findings have to be quantified, and consideration has to be given to the possibility that OKT6-positive cells may be found in lavage fluid in other disorders, and confirmation of the diagnosis should be made by transmission electron microscopy to demonstrate the Langerhans granules in the histiocytosis X cells.

Proposed course: Project completed.

Publications: Chollet, Sylvie, Soler, Paul, Dournovo, Pierre, Richard, Marie S., Ferrans, Victor J., and Basset, Françoise: Diagnosis of Pulmonary Histiocytosis X by Immunodetection of Langerhans Cells in Bronchoalveolar Lavage Fluid. Am J Pathol 115:225-232, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03826-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructural alterations in atrial myocardium of pigs with monensin toxicosis.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John F. Van Vleet, School of Veterinary Medicine, Purdue University

COOPERATING UNITS *(if any)*

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH

Pathology

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Myocardial ultrastructural changes were studied in pigs given toxic doses of monensin, a sodium-selective carboxylic ionophore. Selective injury developed in atrial myocardium, and the resulting lesions were thought to develop from excessive sodium influx and concurrent calcium overload produced by monensin.

f52

Project description:

Objectives: To characterize the ultrastructural features of the lesions which develop selectively in atrial myocardium of pigs given toxic doses of monensin.

Methods employed and major findings: Monensin, a Na^+-selective carboxylic ionophore, produces left atrial damage in pigs given toxic doses. Eight weanling pigs were given mycelial monensin orally (40 mg/kg of body weight) and were euthanatized on day 1, 2, 4 and 16 (two animals at each time interval) for ultrastructural study of the left atrial lesions. On days 1-4, extensive necrosis with contraction bands was present. Rapid macrophagic invasion and phagocytosis of sarcoplasmic debris was seen on day 2 and 4. Missing necrotic myocytes were outlined by persistent "tubes" of external laminae. In some surviving myocytes, sublethal injury was evident on day 1 by mitochondria with condensed conformation and on day 2, 4 and 16 by moderate to marked myofibrillar lysis and sarcoplasmic vacuolation. The myocardial lesions are presumed to result from Na^+ influx and concurrent Ca^{++} overload induced by monensin. Monensin cardiotoxicity in pigs is a unique example of selective injury to the atrial myocardium.

Significance: Administration of monensin to pig produces lesions in the atria but not in the ventricles. This provides a unique model in which to study selective cardiotoxicity. Ultrastructural studies indicate that this toxicity is related to excessive sodium influx and concurrent calcium overload.

Proposed course: Project completed.

Publications: Van Vleet, J. F., and Ferrans, V. J.: Ultrastructural alterations in the atrial myocardium in pigs with acute monensin toxicosis. Am. J. Path. 114: #3, 367-379, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03827-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monensin toxicosis in swine: clinical and pathologic features.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John F. Van Vleet, School of Veterinary Medicine, Purdue University
Harold E. Arstutz, School of Veterinary Medicine, Purdue University
Walter E. Weirich, School of Veterinary Medicine, Purdue University
Alan H. Rebar, School of Veterinary Medicine, Purdue University

COOPERATING UNITS *(if any)*

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH

Pathology Branch

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NHLBI, NIH, Bethesda, MD 20205

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CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The clinical and pathologic features of the toxicosis produced by nomensin, a sodium-selective carboxylic ionophore, were characterized in swine. Clinical signs of toxicosis consisted of lethargy, anorexia, diarrhea and muscle weakness. Elevation of serum creatine phosphokinase and aspartate aminotransferase activity coincided with the development of necrosis in skeletal and cardiac muscle. In the latter, lesions were limited to atrial myocardium. The reason for this selective localization of the cardiac lesions in this animal species is unknown.

854

Project Description:

Objective: To characterize the clinical and pathological features of monensin toxicosis in swine.

Methods employed and major findings: Toxicosis was produced in 10 weanling pigs given monensin, a sodium-selective carboxylic ionophore, (40 mg/kg, orally) to allow making a characterization of the sequential clinical, clinicopathologic, and pathologic alterations on postdosing days 1, 2, 4, 8, and 16. Clinical signs of monensin toxicosis were apparent on day 1 (lethargy, anorexia, diarrhea, and muscle weakness) and had completely regressed by day 6. Electrocardiographic abnormalities were present in 7 of the 10 pigs on day 1, but were infrequently observed thereafter. Serum aspartate aminotransferase and creatine kinase activities were greatly increased on day 1 and moderately increased on days 8 and 16. Elevation of aspartate aminotransferase and creatine kinase activities coincided with the development of acute myocardial and skeletal muscle necrosis. Selective atrial myocardial necrosis appeared in 5 of the 10 pigs. Affected atria were pale, and microscopically, there were necrosis with contraction bands on day 1, macrophagic invasion and lysis on days 2 and 4, and resolving lesions of myocyte loss with persistent myocardial stroma on day 16. Skeletal muscle damage was present in 9 of the 10 pigs as pale areas (more obvious on cut section). Of the muscles examined (n = 7), the frequency and severity of damage were high in diaphragm, vastus lateralis, semitendinosus, triceps, and intercostal; moderate in longissimus lumborum; and low in tongue. Damage appeared to be greatest in muscles that contained a high proportion of type I fibers. In the damaged muscles, there were hyaline necrosis on day 1, macrophagic infiltration on days 2 and 4, and regeneration of necrotic fibers on day 4 and thereafter. With the regeneration, there was a rapid and complete restoration of skeletal muscle structure and function.

Significance: The present study shows that the lesions induced by monensin in swine myocardium are localized in the atria but not in the ventricles. Thus, this model provides a new opportunity to explore differences between atrial and ventricular myocardium in their response to injury.

Proposed course: Project completed.

Publications: Van Vleet, J. F., Amstutz, H. E., Weirich, W. E., Rebar, H., and Ferrans, V. J.: Clinical, clinicopathologic, and pathologic alterations of monensin toxicosis in swine. Am. J. Vet. Res. 44 (No. 8): 1469-1475, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03828-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anthracycline cardiotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A description is presented of the clinical and pathologic features of the cardiotoxicity produced by doxorubicin and daunorubicin, two antineoplastic agents of the anthracycline family. The mechanisms by which these changes develop are reviewed, and new data concerning prevention of this cardiotoxicity are presented. These data show that the most effective means of blocking the cardiotoxicity is administration of ICRF-187 at the same time that anthracyclines are given.

256

Project description:

Objectives: To review the clinical and pathological features of the cardiotoxicity produced by antineoplastic antibiotics of the anthracycline type.

Methods employed and major findings: The practical therapeutic use of daunorubicin and doxorubicin, two antineoplastic drugs of the anthracycline family, is limited by the cardiotoxic effects of these agents. These effects can be subdivided into acute, subacute and chronic depending upon their temporal relationship to the administration of the drugs. The acute effects consist of hypotension, tachycardia and arrhythmias, and develop within minutes after intravenous administration. The subacute effects are characterized by fibrinous pericarditis or myocardial dysfunction, and occur within four weeks of the first or second dose of the drug. The chronic effects become evident only after several weeks or months of treatment, and are manifested by the insidious onset of severe, often fatal congestive heart failure. Anthracyclines induce multiple, complex biochemical changes in myocardium. Among these are: 1) binding of anthracyclines (intercalation) to nuclear and mitochondrial DNA, leading to inhibition of DNA, RNA and protein synthesis, to fragmentation of DNA and to inhibition of DNA repair; 2) binding of anthracyclines to membranes, including Na-K-dependent ATPase activity, calcium transport and intracellular electrolyte balance; 3) inhibition of reactions utilizing coenzyme Q; 4) chelation of divalent cations, including calcium, iron and copper; 5) decrease in the activity of glutathione peroxidase, and 6) promotion of complex peroxidative phenomena by means of reactions mediated by free radicals. It appears that the best protection against anthracycline cardiotoxicity is provided by ICRF-187, a compound that probably functions by blocking the production of free radicals.

Significance: Cardiotoxicity is an important clinical problem in the treatment of neoplasms with anthracyclines. The present study provides new insights into the pathogenesis and prevention of this problem.

Publications: Ferrans, V. J.: Anthracycline cardiotoxicity. IN: Myocardial Injury, Spitzer, John J., (ed.), New York, Plenum Publishing Corp., 1983, pp. 519-532.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03829-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Morphological aspects of cardiac hypertrophy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

None

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.04		

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cardiac hypertrophy involves not only an increase in heart size, but progressive, initially subtle, structural changes in the muscle cells. The ability to describe these changes in detail has contributed greatly to our understanding of their possible functional significance. Structural and functional correlations in the evolving progression of cardiac hypertrophy are examined in this study.

FJF

Project Description:

Objectives: To study various aspects of the morphology of the heart in hypertrophy, with emphasis on ultrastructural changes.

Methods employed and major findings: Cardiac hypertrophy involves not only an increase in heart size but also subtle changes in the ultrastructure of cardiac muscle cells. These changes have been studied in detail only within the past decade, largely as the result of advances in cardiac biopsy techniques and widespread use of open heart surgery. Tissue for electron microscopic study has been obtained from many patients with a variety of acquired and congenital diseases, and comparisons have been made of morphologic findings in patients whose lesions are basically similar except for their duration. This ability to study these morphologic changes as they develop over time has contributed greatly to our understanding of their possible functional significance. Although structural and functional correlations are not always clear, it is evident that the functional state of the hypertrophied heart and the character of its ultrastructural abnormalities evolve in a generally parallel fashion. Functionally, this evolution follows a trajectory involving adaptive growth, a prolonged state of successful adaptation, and, finally, failure. The corresponding ultrastructural changes also can be viewed in terms of three phases. The first involves an increase in the number of contractile units and mitochondria, abnormalities of nuclei and other organelles presumably related to protein synthesis, and changes in membrane systems representing adaptive responses to cellular growth. In the second phase, many of these abnormalities subside, but cells remain larger than normal. The late stage of hypertrophy is associated with loss and disorganization of contractile elements, disruption of cellular and intercellular organization, necrosis, and fibrosis. Neither the mechanisms that stimulate cardiac growth nor the nature of the limits of this growth as an adaptive response are well understood. However, it has become increasingly apparent that ultrastructural findings can provide significant clues to these phenomena.

Significance: An understanding of structural and functional correlations in the progression of cardiac hypertrophy is important in the clinical evaluation of patients with heart disease, particularly with respect to determining when irreversible changes develop in the late stage of hypertrophy.

Publications: Ferrans, V. J.: Morphology of the heart in hypertrophy. Hosp. Pract. 18: 67-78, 1983, and Ferrans, V. J.: Cardiac hypertrophy: morphological aspects. IN: Zak, Radovan, (ed.) Growth of the Heart in Health and Disease. New York, Raven Press, 1984, pp. 187-239.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03830-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ultrastructural myocardial alterations in monensin toxicosis in cattle.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John F. Van Vleet, School of Veterinary Medicine, Purdue University

COOPERATING UNITS *(if any)*
Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The myocardial ultrastructural alterations produced by monensin toxicosis in cattle were characterized. The initial ultrastructural alterations developed from 1 to 11 days after dosing and consisted of extensive sarcoplasmic vacuolization from mitochondrial swelling and lipid accumulation. By day 4, many damaged myocytes also contained dense, compact myelin figures. From day 4 to day 11, myocardial necrosis developed gradually, and the necrotic myocytes appeared either as dense fibers with intact sarcomeres or disrupted fibers with hypercontraction bands. Macrophages invaded the areas of necrosis and lysed fragments of necrotic myocytes. Monensin toxicosis provides a useful model for studies of drug-related myocardial injury.

260

Project Description:

Objectives: To characterize the myocardial ultrastructural alterations produced by monensin toxicosis in cattle.

Methods employed and major findings: The myocardial ultrastructural alterations induced in monensin toxicosis were studied in 20 beef calves weighing approximately 180 kg. Six of the calves were given (orally) 25 mg of monensin/kg of body weight, 7 calves were given 40 mg/kg, and 5 calves were given 40 mg/kg on days 0 and 7; 2 calves were controls. The calves were euthanatized on days 1, 2, 4, 8, 9, and 11 to determine the sequential development of the cardiac lesions. The initial ultrastructural alterations seen in damaged myocytes from days 1 to 11 in all treated calves were extensive sarcoplasmic vacuolation from mitochondrial swelling and lipid droplet accumulation. By day 4, many degenerated myocytes also contained dense compact myelin figures. From day 4 to day 11, myocardial necrosis had developed in degenerated myocytes of the calves that were given the one or two 40 mg/kg doses of monensin. Necrotic myocytes appeared either as dense fibers with intact sarcomeres or disrupted fibers with hypercontraction bands. Lysing necrotic myocytes had disrupted masses of contractile material, pyknotic nuclei, swollen mitochondria with scattered dense matrical granules, and persisting "tubes" of external lamina. Areas of myocardial necrosis had extensive infiltration of macrophages into the interstitium and within the sarcolemmal "tubes" of necrotic myocytes. Macrophages engulfed and lysed fragments of necrotic myocytes. Occasionally, activated fibroblasts were present in the interstitium in areas of myocardial necrosis, but collagen deposition was not observed.

Significnace: Monensin cardiotoxicosis in cattle is characterized by initial myocyte degeneration and delayed onset of myocyte necrosis, and provides a useful model for studies of drug-related myocardial injury.

Proposed course: Project completed.

Publications: Van Vleet, J. F., and Ferrans, V. J.: Ultrastructural myocardial alterations in monensin toxicosis of cattle. Am. J. Vet. Res. 44:, No. 9: 1629-1630, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03831-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ultrastructural immunohistochemistry of elastase-treated elastic fibers.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Antielastin antibody was used, in conjunction with the immonoperoxidase and the immunoferritin methods, to study the localization of elastin in untreated and in elastase-treated elastic fibers of nuchal ligament and aorta of fetal and young adult sheep. The results obtained show: 1) that the immunohistochemical method is sufficiently sensitive to detect amounts of elastin that cannot be identified by ordinary electron microscopic staining; 2) that partially degraded elastic fibers stain more intensely and homogeneously than do normal elastic fibers because their elastin is more loosely arranged and permits the antibody to penetrate, and 3) that the surfaces of microfibrils associated with elastic fibers also contain small amounts of elastin.

f62

Project Description:

Objectives: To use the immunoperoxidase and immunoferritin methods, in conjunction with antielastin antibody, to study the localization of elastin in untreated and elastase-treated elastic fibers of nuchal ligament and aorta of fetal and young adult sheep.

Methods employed and major findings: In conjunction with the immunoperoxidase and immunoferritin methods, antielastin antibody was used to study the locatization of elastin in untreated and elastase-treated elastic fibers of nuchal ligament and aorta of fetal and young adult sheep. In tissues not treated with elastase, the staining reaction for antielastin antibody was localized in the outer zones of the amorphous components and along the surfaces of the microfibrils; the central zones of the amorphous components were unreactive. After mild elastase treatment, incompletely digested amorphous components showed staining both in their central and outer zones, and some of the microfibrils became unreactive. After extensive elastase treatment, small, scattered amorphous components were still found in association with bundles of microfibrils. These components were stained diffusely by the antielastin antibody method but were not detectable by staining with uranyl acetate and lead citrate or with Kajikawa's method for elastin; elastin was not detected on the surfaces of the microfibrils by any of the methods used. These findings were interpreted as indicating that the surfaces of the microfibrils are associated with small amounts of elastin, and that evenly stained amorphous components are composed of elastin which is loosely arranged and allows the penetration of antielastin antibody. These observations support the concept that microfibrils serve an important role as a scaffold for elastin deposition in elastogenesis. Because of their high sensitivity, immunohistochemical methods for detecting elastin are useful to study partially degraded elastic fibers.

Significance: This study demonstrates the practical value of immunohistochemical methods for the histological and ultrastructural detection of normal and partially degraded elastin.

Proposed course: Project completed.

Publications: Fukuda, Y., and Ferrans, V. J.: The electron microscopic immunohistochemistry of elastase-treated aorta in nuchal ligament of fetal and postnatal sheep. J. Histochem. Cytochem. 32: 747-756, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03832-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Protection by ICRF-187 and dimethyl sulfoxide against acetaminophen toxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Antoine N. El-Hage, Division of Drug Biology, Food and Drug Administration, Washington, D.C.

Eugene H. Herman, Division of Drug Biology, Food and Drug Administration, Washington, D.C.

COOPERATING UNITS *(if any)*
Division of Drug Biology, Food and Drug Administration, Washington, D.C.

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Pathology Branch

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The protective activity of ICRF-187 and dimethyl sulfoxide against acetaminophen-induced hepatotoxicity was tested in golden hamsters. Both of these compounds were found to reduce the incidence and the severity of acetaminophen-induced hepatocellular injury.

864

Project Description:

Objectives: To evaluate the protective activity of ICRF-187 and dimethyl sulfoxide against acetaminophen-induced hepatotoxicity. These studies were undertaken because ICRF-187 was found in other studies from this unit to protect against anthracycline-induced cardiotoxicity by a mechanism probably related to the formation of free radicals. Since the hepatocellular necrosis produced by acetaminophen is also thought to be mediated by free radicals, it was considered of interest to determine whether ICRF-187 would also have a protective effect in this system.

Methods employed and major findings: The protective activity of ICRF-187 and dimethyl sulfoxide (DMSO) was tested against acetaminophen-induced hepatotoxicity. Male Syrian golden hamsters injected intraperitoneally between 6 and 8 p.m. for 2 consecutive days with acetaminophen (300 mg/kg) displayed signs of hepatotoxicity as evidenced by increases in enzyme activity and cellular damage. Forty-eight hours after the second acetaminophen dose, the activities of serum glutamic-pyruvic transaminase and alkaline phosphatase were increased compared with levels found in hamsters given only saline. In addition, hepatocellular necrosis was evident in acetaminophen-treated animals. ICRF-187 (300 mg/kg) given 1 hour before acetaminophen attenuated the increases in enzyme activities, and both DMSO (7.3 g/kg) and ICRF-187 reduced the incidence and severity of acetaminophen-induced hepatocellular injury. Both ICRF-187 and DMSO are capable of altering free radical-mediated toxicity in other experimental systems. Whether these compounds reduce acetaminophen-induced liver toxicity by a similar mechanism remains to be determined.

Significance: The finding of a protective effect of ICRF-187 against acetaminophen-induced hepatic necrosis supports the concept that ICRF-187 acts via interference with free radical effects. In other studies, evidence has been presented to indicate that this interference involves chelation of iron needed for the formation of the toxic free radicals.

Proposed course: Project completed.

Publications: El-Hage, A. N., Herman, E. H., and Ferrans, V. J.: Examination of the protective effect of ICRF-187 and dimethyl sulfoxide against acetaminophen-induced hepatotoxicity in Syrian golden hamsters. Toxicology 28: 295-303, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03833-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Influence of vitamin E and ICRF-187 on chronic doxorubicin cardiotoxicity in swine.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Eugene H. Herman, Ph.D., Division of Drug Biology, Food and Drug Administration

COOPERATING UNITS *(if any)*

Division of Drug Biology, Food and Drug Administration, Washington, D. C.

LAB/BRANCH

Pathology

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Ultrastructure

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A comparison was made of the ability of vitamin E and ICRF-187 to protect against the chronic cardiotoxicity induced by doxorubicin in a miniature swine model. Vitamin E afforded only minimal protection against this cardiotoxicity. In contrast, ICRF-187 provided marked protection.

f66

Project Description:

Objectives: To study the potential of vitamin E and ICRF-187 to protect against the cardiotoxicity resulting from chronic administration of doxorubicin.

Methods employed and major findings: Miniature swine (25 to 40 kg) received 6 injections of doxorubicin (1.6 mg/kg) at 3 week intervals (total dose, 9.6 mg/kg), either alone or concurrently with vitamin E (5000 IU/day for 4 days and 1000 units/day for the next 17 days). In a second study, miniature swine received 6 injections of doxorubicin (2.4 mg/kg) at 3 week intervals (total dose 14.4 mg/kg), either alone or 30 min after 12.5 mg ICRF-187/kg (i.p.). All animals were sacrificed 3 weeks after the last injection. The frequency and extent of myocardial lesions (vacuolization and myofibrillar loss) were scored on a scale of 0 to 4+. Such lesions were noted in 8 of 9 pigs given 9.6 mg/kg doxorubicin alone and in all pigs receiving doxorubicin and vitamin E; however, the severity of the lesions was decreased in the latter animals (average score 1.0, compared to 1.8 in those receiving doxorubicin alone). All swine receiving 14.4 mg/kg doxorubicin alone developed myocardial lesions (average score, 2.7); these lesions were severe (3+) in 4 of the animals. In contrast, cardiac lesions were absent in 2, and minimal (average score, 0.7) in 5 of the 7 animals given 14.4 mg/kg doxorubicin in combination with ICRF-187.

Significance: Demonstration of the protective effect of ICRF-187 against doxorubicin-induced cardiomyopathy, an important complication of antineoplastic therapy.

Proposed course: Project completed.

Publications: Herman, E. H., and Ferrans, V. J.: Influence of Vitamin E and ICRF-187 on chronic doxorubicin cardiotoxicity in miniature swine. Lab. Invest. 49: 69-77, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03834-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Specificity of microscopic features of hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

E. Rene Rodriguez, Visiting Fellow, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A review is presented of qualitative and quantitative morphologic findings in the hearts of patients with hypertrophic cardiomyopathy, with emphasis on the significance of the amount of myocardial fiber disarray present in such patients and in patients with other types of heart disease unrelated to hypertrophic cardiomyopathy. A new theory of morphogenesis is proposed to account for the asymmetric septal hypertrophy and the fiber disarray that occur in hypertrophic cardiomyopathy.

f68

Project Description:

Objectives: To define the qualitative and quantitative microscopic features of hypertrophic cardiomyopathy and to present a new hypothesis concerning the morphogenesis of the asymmetric septal hypertrophy that occurs in this disorder.

Methods employed and major findings: A review is presented of the histologic and ultrastructural abnormalities found in the hearts of patients with hypertrophic cardiomyopathy. Evidence is presented to show that myocardial fiber disarray is found in hypertrophic cardiomyopathy as well as in other conditions; however, in the latter it seldom involves more than 5% of the myocytes in transverse sections of ventricular septum. A new theory of morphogenesis is proposed to account for the asymmetric cardiac hypertrophy that characterizes hypertrophic cardiomyopathy. This theory is summarized as follows: 1) hypercontractility is the underlying abnormality affecting cardiac myocytes; 2) this hypercontractility is present during embryonic development and constitutes the stimulus to the inappropriate increase in cardiac mass that takes place in hypertrophic cardiomyopathy; 3) during embryonic development this stimulus results in increased mitotic division (i.e., hyperplasia) rather than in increased size of individual myocytes (i.e., hypertrophy); 4) hyperplasia is preferentially increased in the ventricular septum (perhaps because of the mechanical forces exerted by left and right ventricular contraction on the ventricular septum), thus exaggerating the asymmetric growth that occurs normally in this area during prenatal development; 5) after birth, the septal asymmetry does not regress in patients with hypertrophic cardiomyopathy, as it does in normal individuals, because it is complicated by hyperplasia (i.e., increased numbers of myocytes are already present in the ventricular septum), and 6) the prenatal phase of hyperplasia is followed by a postnatal phase of gradual, progressive hypertrophy in which myocytes enlarge, septal hypertrophy becomes exaggerated, and clinical symptoms eventually develop.

Significance: The morphogenesis of hypertrophic cardiomyopathy is reconsidered in terms of current knowledge of anatomical and clinical aspects of this disorder.

Proposed course: Project completed.

Publications: Ferrans, V. J., and Rodriguez, E. R.: Specificity of light and electron microscopic features of hypertrophic obstructive and nonobstructive cardiomyopathy. Qualitative, quantitative and etiologic aspects. Eur. Heart. J., 4 (Suppl. F): 9-22, 1983.

869

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03835-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cardiac ultrastructure in restrictive cardiomyopathy.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Eloisa Arbustini, University of Padova, Padova, Italy
Carlo Buonanno, University of Verona, Verona, Italy
Gianpaolo Trevi, University of Verona, Verona, Italy
Natale Pennelli, University of Padova, Padova, Italy
Gaetano Thiene, University of Padova, Padova, Italy

COOPERATING UNITS *(if any)*

Department of Pathology, University of Padova, Padova, Italy
Department of Cardiology, University of Verona, Verona, Italy

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH. Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The results of myocardial biopsies in a patient with restrictive cardiomyopathy are presented, together with a review of reported morphologic observations in this disorder. Endocardial and myocardial fibrosis are found, with consequent impairment of diastolic relaxation.

f70

Project Description: Project No. Z01 HL 03835-01 PA

Objective: To characterize cardiac morphological findings in restrictive cardiomyopathy.

Methods employed and major findings: A five-year clinical follow-up and the results of myocardial biopsies are described in a patient with primary restrictive cardiomyopathy. Histologic examination of a right ventricular endomyocardial biopsy taken early in the course of the illness was not contributory. Histologic examination of a left ventricular endomyocardial biopsy 5 years later showed hypertrophy and disarray of the myocytes, thickening of the endocardium and interstitial fibrosis. Connective tissue was compact and regularly oriented in endocardium, but tangled and irregularly oriented in interstitium. From these observations, and from a complete review of the literature on pathologic findings in restrictive cardiomyopathy, it is concluded: 1) that the irregular network of collagen fibrils and elastic fibers limits diastolic relaxation and prevents ventricular dilatation; 2) that the coexisting hypertrophy results from an attempt to maintain normal pump function; and 3) that the myocyte disarray is a consequence of abnormal mechanical forces generated under conditions of severe fibrosis.

Significance: This report provides the first detailed characterization of ultrastructural findings in restrictive cardiomyopathy, and emphasizes the importance of alterations in the arrangement of both the myocytes and the connective tissue in this disorder.

Proposed course: Project completed.

Publications: Arbustini, E., Buouanno, C., Trevi, G., Pennelli, N., Ferrans, V.J., and Thiene, G.: Cardiac ultrastructure in primary restrictive cardiomyopathy. Chest 84: 236-238, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03836-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monensin toxicosis in swine. Dose response and protection studies.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John F. Van Vleet, School of Veterinary Medicine, Purdue University
Harold E. Amstutz, School of Veterinary Medicine, Purdue University
Walter E. Weirich, School of Veterinary Medicine, Purdue University
Alan H. Rebar, School of Veterinary Medicine, Purdue University

COOPERATING UNITS *(if any)*

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH

Pathology Branch

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Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The frequency and severity of the cardiotoxicity produced by monensin, a Na+ selective ionophore, was found to be dose-related in a swine model, using a dose range of 10 to 50 mg/kg of body weight. Sodium selenite and vitamin E provided partial protection against this cardiotoxicity.

872

Project Description: Project No. Z01 HL 03836-01 PA

Objectives: To determine 1) whether the frequency and severity of monensin-induced cardiotoxicity in swine are dose-related, and 2) whether selenium and vitamin E protect against this cardiotoxicity.

Methods employed and major findings: Thirty-two weanling pigs (approx 20 kg each) were used for 2 studies of monensin toxicosis. In the 1st study, 5 groups (designated A through E)of 4 pigs each were given a single oral dose of monensin (10, 20, 30, 40, or 50 mg/kg of body weight) and were observed for 4 days to determine the clinical signs and pathologic alterations. The clinical signs of monensin toxicosis were dose related and included anorexia, diarrhea, lethargy, ataxia, dyspnea, and myoglobinuria; then, death. Clinical signs were not produced by the 10 mg/kg dose of monensin, but mortality resulted within 1 day among pigs given the 50 mg/kg dose. The frequency and severity of monensin-induced myonecrosis were dose-related. Affected pigs had bilaterally symmetrical pale areas of damage in the cranial and caudal thigh muscles, shoulder muscles, and loin muscles. Microscopically, the affected muscles had extensive hyaline necrosis with macrophagic invasion and early regeneration. In a few pigs, there was myocardial necrosis - most often involving the left atrial wall. Myoglobinuria was seen grossly in the pigs given the 50 mg/kg dose that died 1 day after dosing.

In the 2nd study, 6 pigs in 1 group (F) were orally given the 50 mg/kg dose of monensin a day after they were treated with selenium-vitamin E (Se as selenite at dosage of 0.25 mg/kg, and vitamin E as α-tocopherol acetate at dosage of 17 IU/kg; in the other group (G), 6 pigs were treated with placebo (saline solution) before dosing with monensin. Partial protection against monensin toxicosis was provided by Se-E pretreatment as demonstrated by (i) absence of mortality in group F pigs, (ii) smaller increases in serum aspartate aminotransferase and creatine kinase activities in group F pigs than in group G pigs, and (iii) less frequent and less severe clinical signs and lesions.

Significance: The monensin model of skeletal and cardiac muscle necrosis is new and interesting in that the damage is caused by increased influx of sodium, a direct consequence of the sodium-selective carboxylic ionophore effect of this agent.

Proposed course: Project completed.

Publications: Van Vleet, J. F., Amstutz, H. H., Weirich, W. E., Rebar, A. H., and Ferrans, V. J.: Acute monensin toxicosis is swine: Effect of graded doses of monensin and protection of swine by pretreatment with selenium-vitamin E. Am. J. Vet. Res. 44 (No. 8): 1460-1468, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03837-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Bronchoalveolar lavage. Techniques and applications.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Françoise Basset, Faculté Xavier Bichat, INSERM, Paris, France
Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI
Ronald G. Crystal, Chief, Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*
Faculte Xavier Bichat, INSERM, Paris, France
Pulmonary Branch, NHLBI

LAB/BRANCH
Pathology Branch

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Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)
- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study of the cells and fluid obtained by bronchoalveolar lavage has led to considerable progress in many aspects of our knowledge of pulmonary disorders, including pathogenesis and diagnosis. The techniques, methods and results of bronchoalveolar lavage are reviewed in this study, with emphasis on the diagnosis of interstitial lung disorders.

874

Project No. Z01 HL 03837-01 PA

Project Description:

Objectives: To review the techniques employed for bronchoalveolar lavage, to present the results obtained with this technique, and to discuss the diagnostic significance of the findings.

Methods employed and major findings: Bronchoalveolar lavage was originally used in animals for experimental studies of alveolar macrophages; subsequently, it was proposed as a therapeutic technique in patients with pulmonary alveolar proteinosis, using general anesthesia and large amounts of lavage fluid. More recently, the advent of fibroptic bronchoscopic techniques has made it possible to lavage effectively a bronchial segment or subsegment of lung by "wedging" the instrument and utilizing small amounts of lavage fluid. Study of the fluid and cells obtained by this technique has led to considerable progress in many aspects of our knowledge of pulmonary disorders, including pathogenesis and diagnosis, as well as the evaluation of therapeutic results. Bronchoalveolar lavage is now considered an invaluable means of accurately evaluating the inflammatory and immune responses of the lung. Technique of lavage: The trachea and upper airways are anesthetized with zylocaine spray, and a fiberoptic bronchoscope is introduced through the nose into the tracheobronchial tree. Because of technical reasons, the lavage is usually performed on the lingula or on the right middle lobe. Once the bronchoscope is wedged, 20-25 ml of sterile saline, warmed to 37°C, is introduced in the suction port with a syringe. The fluid is immediately pulled back with a negative pressure of 50-100 mm Hg and collected in a container which is maintained a 4°C. The process is repeated several times to instill a total volume of 100 to 150 ml of saline. The fluid collected is strained and a total cell count and a differential cell count are made. Additional preparations are used, as needed, for: 1) immunohistochemical typing of lymphocytes and macrophages by the use of monoclonal antibodies; 2) histochemical staining; 3) transmission and scanning electron microscopy; 4) X-ray microanalysis, and 5) a variety of biochemical and immunological analyses. The results obtained by these techniques are useful not only in establishing and confirming specific diagnoses but also in determining the severity of the disease progress and in following its course and its response to therapeutic interventions.

Significance: Bronchoalveolar lavage is considered an invaluable means of evaluating the inflammatory and immune responses of the lung. Studies of cells and fluid obtained by this technique have contributed considerably to our knowledge of interstitial lung disorders.

Proposed course: Project completed.

Publications: Ferrans, V. J., Basset, F., Fukuda, Y., and Crystal, R. G.: Bronchoalveolar lavage. Techniques and applications. J. Jpn. Med. Soc. Biol. Interface 14: 28-39, 1983.

875

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03838-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ultrastructural alterations in skeletal muscle of pigs with monensin toxicosis.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
John F. Van Vleet, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Large doses of monensin, a sodium-selective carboxylic ionophore, were found to produce polyfocal, monophasic necrosis of skeletal muscle, with type I fiber selectivity, in swine. Regeneration of affected muscles developed early following injury and progressed rapidly to complete restoration of the necrotic muscles without residual fibrosis.

276

Project No. Z01 HL 03838-01 PA

Project Description:

Objectives: To characterize the skeletal muscle lesions produced by monensin in swine.

Methods employed and major findings: Large doses of monensin, a sodium-selective carboxylic ionophore, produced polyfocal, monophasic necrosis of skeletal muscle, with type I fiber selectivity, in swine. To study the sequential ultrastructural alterations involved in this process, 14 weanling pigs were given 40 mg of monensin per kg of body weight and were euthanatized 1, 2, 4, 8, and 16 days later. Myotoxicosis and myoglobinuria were apparent clinically. At necropsy, white, dry areas of necrosis were present in the muscle masses of the anterior and posterior thigh, shoulder and loin. Two patterns of skeletal muscle necrosis were observed on day 1, especially in type I fibers. In fibers exhibiting the first of these patterns, the contractile material was disrupted, forming dense amorphous and filamentous clumps scattered within the persistent sheaths of external lamina (sarcolemmal tubes); the mitochondria were swollen and contained flocculent matrix densities, and the nuclei were pyknotic. Fibers showing the second pattern were uniformly dense, but their sarcoplasm was not disrupted. Sublethally injured fibers were also observed and had focal myofibrillar lysis. On days 2 and 4, the necrotic muscle had marked infiltration of macrophages in the interstitium and within sarcolemmal tubes. Rapid resolution of the fiber necrosis occurred by phagocytosis of the sarcoplasmic debris. Regeneration of affected muscles developed early following injury and progressed rapidly to complete restoration of the necrotic muscles without residual fibrosis. Regeneration was initiated on day 1 by activation of satellite cells to form presumptive myoblasts; on days 4 and 8 these cells showed evidence of fusion, forming myotubes to restore the necrotic fibers.

Significance: This work provides a new model system for the study of necrosis produced directly by excessive influx of sodium into skeletal muscle fibers.

Proposed course: Project completed.

Publications: Van Vleet, J. F., and Ferrans, V. J.: Ultrastructural alterations in skeletal muscle of pigs with acute monensin myotoxicosis. Am. J. Pathol. 114: #3, 461-471, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03839-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prevention of doxorubicin cardiotoxicity by liposomal encapsulation of the drug.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Eugene H. Herman, Food and Drug Administration, Washington, D.C.
James A. Vick, Food and Drug Administration, Washington, D.C.
A. Rahman, Vincent T. Lombardi Cancer Research Center, Georgetown University School of Medicine, Washington, D.C.
Philip S. Schein, Vincent T. Lombardi Cancer Research Center, Georgetown University School of Medicine, Washington, D.C.

COOPERATING UNITS *(if any)*

Dividion of Drug Biology, Food and Drug Administration, Washington, D.C.
Division of Medical Oncology, Vincent T. Lombardi Cancer Research Center, Georgetown University School of Medicine, Washington, D. C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies were made to determine whether chronically administered, liposom-encapsulated doxorubicin would be less toxic than the free drug when given to beagle dogs according to a model system previously developed in this unit. Liposomal encapsulation of doxorubicin markedly decreased cardiac and other toxic effects elicited by free doxorubicin. Whether this advantage can be translated into antieffective antineoplastic therapy will need further evaluation.

878

Project Description:

Objectives: To determine whether chronically administered, liposome-encapsulated doxorubiein is less cardiotoxic than the free drug when given to beagle dogs according to a model system previously developed in this unit.

Methods employed and major findings: A number of cytotoxic drugs have been encapsulated into liposomes as a means of enhancing their pharmacologic activity and reducing their systemic toxicity. The present study was initiated to determine whether chronically administered liposome-encapsulated doxorubicin would be less toxic than the free drug. Doxorubicin was prepared in positively charged cardiolipin liposomes and 1.75 mg/kg was given i.v. to each of 5 beagle dogs. A second group received the free drug at 1.75 mg/kg. Additional animals received i.v. injections of either doxorubicin free liposomes or saline. All substances were given at 3-week intervals and the experiment ended 1 week after the 7th injection (12.25 mg/kg total dose). A temporary reduction in food consumption was noted during the first few days after the administration of either form of doxorubicin. The effect was more severe in the dogs given free doxorubicin, and body weight decreased significantly only in this group of animals. Three dogs given free doxorubicin died or were killed before the end of the study because they were in poor condition. Lesions consisting mainly of vacuolization and myofibrillar loss were noted in the hearts of all 5 dogs given free doxorubicin. The severity of the lesions ranged from 2 to 4 (av. 3.4). In contrast, no abnormalities were found in any of the hearts from dogs given the liposomal doxorubicin. The most obvious general toxic effect caused by administration of free doxorubicin was alopecia, which was entirely prevented when doxorubicin was encapsulated into liposomes. At the dosage regimen utilized, liposomal doxorubicin and free doxorubicin exerted comparable degrees of bone marrow suppression.

Significance: This study shows that liposomal encapsulation of doxorubicin constitutes a practical means of giving this drug and results in a decrease in cardiac and other toxic effects elicited by free doxorubicin. Whether this advantage can be translated into effective antineoplastic activity will need further evaluation.

Proposed course: Project completed.

Publications: Herman, E. H., Rahman, A., Ferrans, V. J., Vick, J. A., and Schein, P. S.: Prevention of chronic doxorubicin cardiotoxicity in beagle dogs by liposomal encapsulation. Cancer Res. 43: 5427-5432, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03840-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
ICRF-187 reduction of chronic anthracycline cardiotoxicity.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Eugene H. Herman, Division of Drug Biology, Food and Drug Administration, Washington, D. C.

COOPERATING UNITS *(if any)*
Division of Drug Biology, Food and Drug Administration, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The potential protective effect of ICRF-187, a bisdiketopiperazine compound (1,2-bis(3,5-dioxopiperazin-1-yl(propane) against the chronic cardiomyopathy induced by daunorubicin and doxorubicin, was evaluated in rabbits, beagle dogs and miniature pigs. The results obtained show that ICRF-187 reduces markedly the severity of this cardiomyopathy without producing undesirable side effects.

FFO

Project Description:

Objectives: To evaluate the protective effect of ICRF-187 against anthracycline cardiomyopathy in rabbits, dogs and miniature swine.

Methods employed and major findings: The potential protective effect of ICRF-187 against the chronic cardiomyopathy induced by daunorubicin and doxorubicin was examined in rabbits, beagle dogs and miniature pigs. Rabbits were given 3.2 mg/kg daunorubicin alone or 30 minutes after 12.5 or 25 mg/kg ICRF-187 at 3 week intervals (5 injections). Beagle dogs were given doxorubicin (1.0 mg/kg) either alone or 30 minutes after 12.5 mg/kg ICRF-187 at weekly intervals (15 injections). Miniature pigs were given 6 i.v. injections of 2.4 mg/kg doxorubicin alone or 30 minutes after 12.5 mg/kg ICRF-187 at 3 week intervals. Lesions consisting mainly of vacuolozation and myofibrillar loss were noted in the hearts of all 12 rabbits, 6 dogs and 6 miniature pigs given the anthracyclines alone. In contrast, no abnormalities were found in 4 of 12 rabbits, 4 of 6 dogs and 2 of 7 miniature pigs pretreated with ICRF-187. The remaining rabbit, dog and miniature pig hearts from the ICRF-187 pretreatment groups had minimal alterations. Serum iron concentration was decreased in all animals treated with the anthracyclines with or without ICRF-187. Anthracycline treatment was associated with decreases in RBC, WBC and hemoglobin concentration. ICRF-187 pretreatment did not prevent or enhance this activity. Thus, concurrent administration of ICRF-187 offers a means of reducing chronic daunorubicin and doxorubicin cardiac toxicity in several species of animals.

Significance: These studies show that ICRF-187 offers a practical means of reducing the cardiotoxicity of anthracyclines in several different animal species. The animal models developed during the course of these studies are useful to study the mechanisms of cardiotoxicity of anthracyclines.

Proposed course: Project completed.

Publications: Herman, E. H., and Ferrans, V. J.: ICRF-187: Reduction of chronic daunorubicin and doxorubicin cardiotoxicity in rabbits, beagle dogs and miniature pigs. Drugs Exptl. Clin Res. IX: 483-490, 1983.

FY1

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03841-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of alveolar septa in fetal sheep lung.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI
Ronald G. Crystal, Chief, Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*

Pulmonary Branch, NHLBI, NIH

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The morphogenesis of alveolar septa in sheep lung was studied by means of light microscopic, electron microscopic and immunohistochemical techniques. The concept is presented that alveolar septa develop from primordia which first develop as groups of fibroblasts in the area subjacent to the epithelium and are associated with elastic fibers and collagen fibers; these structures undergo a complex process of differentiation of cells and of organization of connective tissue. Immunohistochemical staining for elastin provided a very useful technique to follow this process.

f12

Project Description:

Objectives: To study in detail the morphogenesis of alveolar septa in sheep lung by means of light microscopic. electron microscopic and immunohistochemical techniques.

Methods employed and major findings: The morphogenesis of pulmonary alveolar septa in the sheep was studied by means of light microscopy, transmission electron microscopy and techniques of light microscopic immunohistochemistry for the detection of elastin. The primordia of alveolar septa developed in the glandular stage in areas subjecent to the epithelium, and formed alveolar septa by protruding into the glandular lumina. In their earliest stage, the primordia consisted of groups of fibroblasts, which were associated with elastic fibers and collagen fibers and were surrounded by epithelial basement membrane and by more immature fibroblasts. The fibroblasts in the primordia subsequently became myofibroblasts or smooth muscle cells. In the alveolar zone of the glands, elastic fibers were exclusively found in the primordia of alveolar septa in early developing lung. In early developing lung, wavy, thickened epithelial basement membranes were found in the regions of the glands which eventually underwent considerable expansion of their surface areas, especially in the primordia of alveolar septa and the bifurcations in the alveolar zones. Areas of fusion of the basement membranes of capillary endothelial cells and epithelial cells in the alveolar zone were found after the formation of the primordia of alveolar septa was accomplished; these areas of fusion were not found in the primordia themselves, but in regions between the primordia. Epithelial cell flattening and differentiation occurred after the formation of the primordia of alveolar septa and flattening was first observed in the areas of the primordia and the bifurcations of the alveolar zones.

Significance: This report presents a new concept of the morophogenesis of pulmonary alveolar septa and shows that immunohistochemical staining for elastin constitutes a very useful technique for studying pulmonary morphogenesis.

Proposed course: Project completed.

Publications: Fukuda, Y., Ferrans, V. J., and Crystal, R. G.: The development of alveolar septa in fetal sheep lung. An ultrastructural and immunohistochemical study. Am. J. Anat. 167: 405-439, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03842-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Clinical and pathologic alterations in acute monensin toxicosis in cattle.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John F. Van Vleet, School of Veterinary Medicine, Purdue University
Harold E. Amstutz, School of Veterinary Medicine, Purdue University
Walter E. Weirich, School of Veterinary Medicine, Purdue University
Alan H. Rebar, School of Veterinary Medicine, Purdue University

COOPERATING UNITS *(if any)*
Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.04		

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monensin, a polyether antibiotic, acts as a sodium-selective ionophore, and when given in large doses it produces toxicosis with myocardial and skeletal muscle necrosis. The monensin model of necrosis presents certain unique features, related to the fact that the primary effect of this agent is to increase sodium entry into cells. This study provides a detailed characterization of the clinical and pathologic features of the cardiac necrosis induced by monensin.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description:

Objectives: To characterize the clinical and pathological features of monensin toxicosis in cattle.

Methods employed and major findings: Twenty beef calves weighing approximately 180 kg were allotted to 3 groups. In group A, 6 calves were given 25 mg of mycelial monensin/kg of body weight orally and were evaluated at 1, 2, and 4 days for clinical, ECG, clinicopathologic, and pathologic alterations. In group B, 7 calves were given a single dose of monensin (40 mg/kg) and 5 were given a 2nd 40 mg/kg dose on day 7; calves were evaluated at days 1, 2, 4, 7, 8, 9, and 11. In group C, 2 calves served as controls. Monensin-treated calves developed anorexia, diarrhea, and lethargy after day 1. One group B calf died on day 7 with lesions of congestive heart failure. Electrocardiographic abnormalities were not observed in group A calves; in group B, prolongation of Q-T and QRS intervals occurred from days 2 to 11 and first degree heart block was seen from days 7 to 11. Clinicopathologic alterations included: increased serum activities of aspartate aminotransferase and creatine kinase in group B calves after day 2; decreased serum K^+, Na^+, and Ca^2 concentrations in both groups, and postdosing occurrence of leukocytosis. Calves were euthanatized sequentially and the lesions of monensin toxicosis were present in the heart, skeletal muscles, and rumen in groups A and B. Disseminated pale yellowish-brown areas of necrosis were present in the ventricular myocardium of 6 of 12 group B calves. Gross lesions were not present in the skeletal muscles or rumen. Microscopically, the myocardial and skeletal muscular lesions were characterized by sarcoplasmic vacuolation from mitochondrial swelling and lipid accumulation in calves killed after day 1 in groups A and B, and by myocardial necrosis with contraction bands, but without calcification, in group B calves killed by day 4. Acute rumenitis was present in groups A and B calves.

Significance: This study indicates that the myotoxic effects of monensin may be related to its action as an ionophore producing altered intracellular ion concentrations and initiating degeneration and necrosis in striated muscle fibers.

Publications: Van Vleet, J. F., Amstutz, H. E., Weirich, W. E., Rebar, A. H., and Ferrans, V. J.: Clinical, clinicopathologic, and pathologic alterations in acute monensin toxicosis in cattle. Am. J. Vet. Res. 44, (No. 11): 2133-2144, 1983.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03843-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Crystalline structures in skeletal muscle fibers.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure

INSTITUTE AND LOCATION

NHLBU, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

A discussion is presented, with emphasis on differential diagnosis, of crystalline structures found in skeletal muscle biopsies. The most important possibilities to be considered include: nemaline rods; crystals composed of other proteins of the contractile system; intramitochondrial crystals, and viral crystals.

ff b

Project Description: Project No. Z01 HL 03843-01 PA

Objectives: To discuss the differential diagnosis of crystalline structures found in skeletal muscle biopsies.

Methods employed and major findings: The possibilities that must be considered in the interpretation of crystalline structures found in skeletal muscle biopsies include: 1) nemaline rods; 2) crystals composed of other proteins of the contractile system; 3) intramitochondrial crystals, and 4) viral crystals. Viral crystals, which have been observed in skeletal muscle in a number of types of viral infections, appear as well defined arrays of viral particles. Glycogen granules can become organized into highly ordered arrays that can be confused with viral crystals, and can be distinguished from the latter by appropriate electron microscopic histochemical stains. Intramitochondrial crystals have been reported in muscle in a wide variety of conditions: in some instances they do not have a relationship to the mitochondrial membranes, which may have disappeared, leaving the crystals free in the cytoplasm. Calcific deposits in skeletal, smooth and cardiac muscle can be associated with mitochondria, or can be present in the main cytoplasmic compartment, but also can occur in connective tissue, usually in the form of highly electron-dense, needle-like particles of hydroxyapatite. Nemaline rods are thought to be composed of α-actinin. The spectrum of "nemaline myopathies" is ample, and it is thought that variations in the conditions of fixation, dehydration, and embedding of tissues, and in instrument calibration, can account for many of the reported differences in the periodicity of the nemaline "rods". Other contractile proteins such as tropomyosin, meromyosin, actin and actomyosin are capable of forming crystal-like arrays, the morphological characteristics of which vary accoridng to the physicochemical conditions (pH, ionic strength, temperature, etc.) under which they are induced to develop in vitro. It remains uncertain to what extent this can occur in vivo in muscle tissues.

Significance: Crystalline structures are a relatively frequent finding in skeletal muscle biopsies, and the present study provides some useful guidelines for evaluating their nature and diagnostic significance.

Proposed course: Project completed.

Publications: Neustein, H. B., Pepkowitz, S. H., Erlandson, R. A., and Ferrans, V. J.: Crystalloid structures in muscle biopsies. Comments by the panel. Ultrastruct. Pathol. 6 (No. 1): 105-108, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03844-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The histiocytoses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Françoise Basset, Faculté Xavier Bichat, INSERM, Paris, France
Christian Nezelof, Hôpital Necker-Enfants Malades, INSERM, Paris, France

COOPERATING UNITS *(if any)*
Faculté Xavier Bichat and Hôpital Necker-Enfants Malades, INSERM, Paris, France

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.04		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A review is presented of the clinical, histologic and electron microscopic features of the different types of histiocytoses, diseases which have in common the proliferation of cells of the mononuclear phagocyte system.

fff

Project Description: Project No. Z01 HL 03844-01 PA

Objectives: To define and present in detail the characteristics of the histiocytoses, with emphasis on the histologic, immunohistochemical and ultrastructural features which are useful in establishing their diagnosis.

Methods employed and major findings: The histiocyteses are chronic diseases characterized clinically by multiple organ involvement and pathologically by a proliferation of histiocytic cells. In this review, they are classified into the following four large groups on the basis of their clinical and pathological features: 1) secondary histiocytoses, which develop as reactions to chronic inflammation or immune disorders; 2) storage histiocytoses, which are manifested by storage phenomena of exogenous or endogenous origin; 3) Langerhans cell histiocytoses, which are characterized by the presence of numerous cells related to epidermal Langernans cells,, which include the various forms of histiocytosis X (Hand-Schüller-Christian disease, Letterer-Siwe disease, eosinophilic granuloma of bone and pulmonary histiocytosis X), and 4) malignant (neoplastic) histiocytoses.

Significance: This work provides guidelines for the differential diagnosis of the histiocytoses, diseases which present important clinical problems, particularly with respect to the diagnosis of pulmonary histiocytosis X.

Proposed course: Project completed.

Publications: Basset, F., Nezelof, C., and Ferrans, V. J.: The histiocytoses. Pathol. Annu. 18, Part II: 27-78, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03845-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Asymptomatic Sinus of Valsalva Aneurysm Causing Right Ventricular Outflow

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

Carole A. Warnes, Visiting Fellow, Pathology Branch, NHLBI

Barry J. Maron, Cardiology Branch, NHLBI
Michael Jones, Surgery Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

XX ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Sinus of Valsalva aneurysm (SVA) unassociated with ventricular septal defect does not produce symptoms of cardiac dysfunction until the wall of the SVA ruptures or the aneurysm itself obstructs right ventricular (RV) outflow. If rupture occurs, usually symptoms appear abruptly and congestive heart failure progresses rapidly thereafter. Obstruction to RV outflow by bulging of the SVA into the RV outflow tract has been demonstrated hemodynamically in only 2, possibly 3, patients. The patient in this report is unique because the degree of RV outflow tract obstruction by a SVA was observed to increase with time and symptoms of cardiac dysfunction never occurred despite rupture of the wall of the SVA.

890

Project Description:

Sinus of Valsalva aneurysm (SVA) unassociated with ventricular septal defect does not produce symptoms of cardiac dysfunction until the wall of the SVA ruptures or the aneurysm itself obstructs right ventricular (RV) outflow. If rupture occurs, usually symptoms appear abruptly and congestive heart failure progresses rapidly thereafter. Obstruction to RV outflow by bulging of the SVA into the RV outflow tract has been demonstrated hemodynamically in only 2, possibly 3, patients. The patient in this report is unique because the degree of RV outflow tract obstruction by a SVA was observed to increase with time and symptoms of cardiac dysfunction never occurred despite rupture of the wall of the SVA.

Publication:

Warnes, C A., Maron, B. J., Jones, M., Roberts, W.C.: Asymptomatic Sinus of Valsalva Aneurysm Causing Right Ventricular Outflow Obstruction Before and After Rupture. American Journal of Cardiology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03846-01 PA

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Aneurysm (Redundancy) of the Atrial Septum (Fossa Ovale Membrane) and Prolapse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

XX (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This report described patients along with previous echocardiographic evidence indicate an association between aneurysm of the fossa ovale membrane of the atrial septum and prolapse of the mitral valve. The presence of aneurysm of a sinus of Valsalva and congenital anomalies of both aortic and pulmonic valves in 1 of the 2 patients suggests a developmental association of these additional anomalies with atrial septal aneurysm and MVP.

892

Project Description:

This report described patients along with previous echocardiographic evidence indicate an association between aneurysm of the fossa ovale membrane of the atrial septum and prolapse of the mitral valve. The presence of aneurysm of a sinus of Valsalva and congenital anomalies of both aortic and pulmonic valves in 1 of the 2 patients suggests a developmental association of these additional anomalies with atrial septal aneurysm and MVP.

Publication:

Roberts, W. C.: Aneurysm (Redundancy) of the Atrial Septum (Fossa Ovale Membrane) and Prolapse (Redundanc) of the Mitral Valve: A Recently Recognized Association. American Journal of Cardiology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03847-01 PA

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Formation of New Coronary Arteries Within a Previously Obstructed Epicardial

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

Renu Virmani, Vanderbilt University, Nashville, TN

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Small vascular channels often develop within previously obstructed arteries, but usually such channels are quite small and the arterial lumen consequently remains quite narrowed. That large channels consisting of muscular arteries and capable of carrying sizable quantities of blood may develop in previously obstructed larger muscular arteries is not a well recognized observation. Such was the case in 2 patients described in this paper.

Each patient in the report had healed transmural left ventricular scars and severe narrowing of 1 major epicardial coronary artery without any narrowing of the other major arteries. Numerous small muscular arteries had formed in the narrowed lumens of a previously obstructed artery. Patient #1 had an embolus to the left anterior descending coronary artery during active infective endocarditis involving the aortic valve and the embolic material subsequently organized to form the new small muscular arteries within the larger muscular artery. Patient #2 presumably also had thrombus or embolus in the ramus branch of the left main coronary artery but its source was not determined; it too subsequently organized with development of multiple new muscular arteries within the obstructed artery. These 2 patients demonstrate that the human body is capable of forming normal coronary arteries within large epicardial coronary arteries.

Had coronary angiogram been performed in each of these 2 patients after healing of the acute myocardial infarction almost surely the artery containing the newly formed arteries within the previously obstructed artery would have appeared angiographically normal. Thus, formation of multiple new small muscular arteries within previously obstructed large muscular coronary arteries is a mechanism of producing angiographically normal coronary arteries after healing of acute myocardial infarction.

894

Project Description:

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Publication:

Roberts, W. C. and Virmani, R.: Formation of New Coronary Arteries Within a Previously Obstructed Epicardial Coronary Artery (Intraarterial Arteries): A Mechanism for Occurrence of Angiographically Normal Coronary Arteries After Healing of Acute Myocardial Infarction. American Journal of Cardiology, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03848-01 PA

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anomalous Origin of the Left Anterior Descending Coronary Artery

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts, Chief, Pathology Branch, NHLBI

Renu Virmani, Vanderbilt University, Nashville, TN

COOPERATING UNITS *(if any)*

LAB/BRANCH: Pathology Branch, NHLBI

SECTION:

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, MD

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
416 hours	416 hours	

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Origin of both right and left main (LM) coronary arteries from the pulmonary trunk (PT) rarely allows survival for more than 2 weeks after birth. Origin of the LM coronary artery from the PT with origin of the right coronary artery from the aorta allows longer survival but usually (80%) for no more than 1 year after birth. Origin of the right coronary artery from the PT and the LM coronary artery from the aorta, in contrast, allows survival into adulthood and indeed maybe even a normal life span. Because the LM coronary artery is equivalent to 2 major coronary arteries, whenever the LM arises from PT, whether in association with the right or when isolated, survival is short. When 1 major branch of the LM, however, arises from the PT and both the right and the other major LM branch arise from aorta survival should be similar to that in patients in whom the right coronary artery arises from the PT and the LM from the aorta. This report describe a man in whom the left anterior descending (LAD) coronary artery arose from the PT and the right and LC coronary arteries, from aorta.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description:

Origin of both right and left main (LM) coronary arteries from the pulmonary trunk (PT) rarely allows survival for more than 2 weeks after birth. Origin of the LM coronary artery from the PT with origin of the right coronary artery from the aorta allows longer survival but usually (80%) for no more than 1 year after birth. Origin of the right coronary artery from the PT and the LM coronary artery from the aorta, in contrast, allows survival into adulthood and indeed maybe even a normal life span. Because the LM coronary artery is equivalent to 2 major coronary arteries, whenever the LM arises from PT, whether in association with the right or when isolated, survival is short. When 1 major branch of the LM, however, arises from the PT and both the right and the other major LM branch arise from aorta survival should be similar to that in patients in whom the right coronary artery arises from the PT and the LM from the aorta. This report describe a man in whom the left anterior descending (LAD) coronary artery arose from the PT and the right and LC coronary arteries, from aorta.

Publication:

Roberts, W. C. and Virmani, R.: Anomalous Origin of the Left Anterior Descending Coronary Artery From the Pulmonary Trunk With Origin of the Right and Left Circumflex Coronary Arteries From the Aorta. American Journal of Cardiology, in press.

Annual Report of the Pulmonary Branch
National Heart, Lung and Blood Institute
October 1, 1983 through September 30, 1984

The research of the Pulmonary Branch centers on diseases of the alveolar structures, the site in the body at which gas exchange takes place between the air and blood. Two categories of common diseases are investigated, the interstitial lung disorders, diseases characterized by thickening of the alveolar walls, and the destructive lung disorders, disorders characterized by disruption and loss of the alveolar walls. Both categories of disease are chronic inflammatory disorders where inflammatory cells accumulate in the lower respiratory tract and cause the changes that characterize each disease. Because it is the alveolar structures that are involved, the inflammation is usually referred to as the "alveolitis" of the disease. In this context, it is an understanding of the alveolitis that is central to understanding these diseases.

The interstitial lung disorders are a group of heterogeneous, chronic diseases of the alveolar structures characterized by an alveolitis, changes in alveolar epithelial, endothelial, and mesenchymal cells, and alterations in the alveolar connective tissue matrix referred to as "fibrosis". The interstitial disorders of known etiology represent 35% of all interstitial disorders and include the inhalational disorders (e.g., the pneumoconioses, hypersensitivity pneumonitis) as well as the disorders resulting from radiation, drugs and poisons. There are many interstitial lung disorders of unknown etiology but some of the most commonly encountered are sarcoidosis and idiopathic pulmonary fibrosis (IPF). The current concepts of the pathogenesis of all of the interstitial diseases suggest that it is the specific inflammatory and immune processes comprising the alveolitis that modulate the changes to the parenchymal cells and connective tissue matrix that define the physiologic abnormalities that characterize each disorder.

The destructive lung disorders are chronic diseases of the lower respiratory tract characterized by the loss of the alveolar structures. The current concepts of the pathogenesis of these disorders are defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in normal lung, proteases (e.g., neutrophil elastase, an enzyme that can destroy most lung connective tissue components) released by inflammatory cells within the alveolar structures, are balanced by antiproteases (e.g., alpha 1-antitrypsis, a circulating antielastase that diffuses into the lung). In the destructive lung diseases, there is increasing evidence that there is an imbalance such that a chronic alveolitis presents an elastase burden that overpowers an insufficient antielastase protective screen, thus causing connective tissue destruction and loss of the alveolar structures.

The technique of bronchoalveolar lavage permits direct access to the epithelial surface of the lower respiratory tract where inflammatory cells and relevant molecules in the epithelial lining fluid can be easily and repetitively sampled in normals and patients with interstitial disorders. To accomplish bronchoalveolar lavage, local anesthesia is administered to the upper respiratory tract and a fiberoptic bronchoscope is gently wedged into a distal bronchus. Sterile saline, usually in 5, 20 ml aliquots in 1 to 3 sites, is infused into the bronchoscope and then suctioned back, thus sampling the epithelial lining fluid of the lower respiratory tract.

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In the normal lung, the majority of the inflammatory cells in the alveolar structures are alveolar macrophages, cells derived from the mononuclear phagocyte system. The remaining inflammatory cells are lymphocytes. Recent studies have demonstrated that the usual techniques of utilizing a cytocentrifuge to prepare cells recovered by lavage for analysis underestimated the actual proportion of lymphocytes present. With a millipore filtering method, however, absolute quantitation of the cell type can be accomplished. With this approach, studies of normal individuals reveals that, on the average, 83% of cells are alveolar macrophages and 17% are lympocytes.

The normal nonsmoking human has small numbers of alveolar macrophages per alveolus (approximately 50 to 70). Alveolar macrophages are thought to be the "directors" of inflammatory processes in the lower respiratory tract. In addition to being phagocytic, these cells can direct lymphocytes to proliferate, suppress immune processes, and recruit inflammatory cells such as neutrophils. Using monoclonal antibodies directed toward cell surface antigens of cells of the mononuclear phagocyte series it has been possible to demonstrate that the population of alveolar macrophages can be categorized into subpopulations depending on their cell surface characteristics. For example, anti-DR (an antibody directed against a non-polymorphic HLA-DR epitope) is positive in or greater than 90% of normal alveolar macrophages, while ODT9 (an antibody directed against the transferrin recptor) is positive on approximately 30% of alveolar macrophages, and OKM1 (an antibody that recognized the C3Bi receptor) is positive in 15 to 35% of normal alveolar macrophages. At least part of these subpopulation differences appears to be related to the process of maturation of mononuclear phagocytes. It is known that alveolar macrophages are derived from blood monocytes. As a model of monocyte to alveolar macrophage differentiation, blood monocytes are placed in culture and evaluated for cell surface characteristics over time. Whereas blood monocytes are OKT9- (i.e., they do not express the transferrin receptor), as they "mature" in culture, the transferrin receptor is expressed. Likewise, wheras approximately 90% normal blood monocytes express the cell surface antigens 63D3, MØP-9, mØS-1 and MØS-39, in culture they rapidly lose these cell surface characteristics, consistent with the findings that only 5 to 40% of normal alveolar macrophages express the same antigens.

Normal functioning of the immune system requires communication between the mononuclear phagocyte system and T-lymphocytes. One means by which this occurs is through a mediator, interleukin-1, a 16,000 dalton protein produced by mononuclear phagocytes that plays an important role in the early steps of T-cell proliferation in response to antigens. Recent studies have demonstrated that human alveolar macrophages release far less interleukin-1 (approximately 20% with a standard stimulus) than do human blood monocytes. Since the lung is heavily exposed to antigens, the fact that a critical mediator may be rate limiting may be one mechanism by which antigenic responses are controlled in the lower respiratory tract. Consistent with this observation, normal human alveolar macrophages "present" antigens to autologous blood or lung T-lymphocytes in a manner that results in approximately one-quarter of the T-cell proliferation as do autoloqous blood monocytes. Thus, if a population of normal blood monocytes and T-lymphocytes were exposed to the same quantity of antigen as normal alveolar macrophages and T-lymphocytes, the T-cells exposed to the monocytes would proliferate to a much greater extent.

Classical concepts of how mononuclear phagocytes accumulate in areas of inflammation suggest that all are recruited from blood monocytes that are called to

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the site of inflammation. However, recent studies demonstrated that human alveolar macrophages are capable of replicating and they do so in many inflammatory lung disorders. Thus, the increase in macrophage numbers found in these diseases probably represent, in part, local proliferation together with recruitment from blood monocytes. While the mechanisms inducing this macrophase replication are not known, growth factors in the local inflammatory milieu are likely responsible. In this regard, the fact that alveolar macrophages can proliferate is consistent with the knowledge that transferrin is required for cell proliferation and that a significant proportion of normal alveolar macrophages express the transferin receptor.

The normal human lung contains approximately 9 to 12 lymphocytes per alveolus. Using monoclonal antibodies directed toward cell surface antigens, studies have demonstrated that approximately 90% are T-lymphocytes (OKT3+, Leu 4+) and 2% are B-lymphocytes (B1+). Of the T-cells, approximately 65% have surface antigens characteristic of "helper" cells (OKT4+, Leu 3+) while 30-35% are characteristic of "suppressor/cytotoxic" cells (OKT8+, Leu 2+). Very few of the 3+ or 2+ normally express surface antigens suggesting they are activated (e.g., DR, DS, and 4F2 antigens). Consistent with this observation, in vitro evaluation of normal lung T-cells shows that they are not spontaneously releasing mediators such as interferon-gamma, interleukin-2, or monocyte chemotactic factor. Although 2% of the lympocytes on the epithelial surface of the lower respiratory tract are B-cells, evaluation of these cells demonstrates that, unlike normal blood B-cells, they are not spontaneously releasing immunoglobulins. The reason for this is not clear, but likely relates to the fact that the normal milieu of the alveolar epithelial surface suppresses inflammatory processes. Consistent with this concept, morphologic studies of the lymphocytes recovered from the normal lower respiratory tract show that approximately 10% are "large granular lymphocytes", characteristics of natural-killer cells (cells of the lymphocyte series that are spontaneously cytotoxic for tumor cells). Interestingly, however, there is very little natural killer cell activity among the lymphocyte populations of the lung. This lack of lung natural killer cell activity may be due, in part, to the ability of human alveolar macrophages and alveolar epithelial living fluid to suppress natural killer cells. However, when interleukin-2, the T-cell growth factor, is added to a population of lung lymphocytes, natural killer activity is expressed. As a corollary to this observation, lung lymphocytes in active sarcoidosis do have natural killer cell activity, a finding consistent with the knowledge that lung T-cells in active are sarcoid are spontaneously releasing interleukin-2. Furthermore, in vitro studies with natural killer cells have demonstrated that chrysotile asbestosis suppress natural killer activity, an observation consistent with the knowledge that long term exposure to high levels of chrysotile asbestos increases the risk of lung cancer in cigarette smokers.

In addition to inflammatory cells, the technique of bronchoalveolar lavage permits the sampling of the ephthelial lining fluid (ELF) and thus allows analysis if the various macromolecules that play a role in mediating inflammatory and immune processes as well as defending the lung. Some of these molecules include immunoglobulins, complement components, arachadonic acid metabolites (e.g., prostaglandins, leukotrienes), surface active material, antiproteases and antioxidants. It is difficult to quantify the macromolecules in this fluid in absolute terms because the fluid returned is mixed with the large amounts of saline (usually in a 100 to 1 ratio) used for the lavage procedure. One approach is to compare the amount of each component to albumin, a method that permits

comparison to values in plasma. Recent studies have demonstrated that by quantifying the amount of urea present in the returned lavage fluid and comparing that to a concentration of urea in the plasma, an absolute estimate of epithelial lining fluid (the "apparent urea volume of epithelial lining fluid") can be determined. It is now possible, therefore, to quantify in absolute terms the amounts of macromolecules in the epithelial lining fluid and to compare these in disease states.

Proteases, enzymes capable of destroying proteins, particularly those comprising the connective tissue matrix that provide the supporting network of the alveolar walls, are released in the lower respiratory tract by inflammatory cells. The two major antiproteases of human alveolar ELF are alpha 1-antitrypsin (a1 AT) and alpha 1-antichymotrypsis (a1ACT). Both are produced primarily in the liver; they are secreted into the blood where, because of their molecule weight (50-60,000 daltons) are capable of diffusing through the alveolar walls. The concentration of a1AT in normal plasma is 150-250 mg/dl and that of a1ACT is 35-60 mg/dl; in ELF the concentrations of both are approximately 10% of that in plasma. a1AT is the major antiprotease capable of defending against neutrophil elastase, an omniverous protease capable of degrading all connective tissue components. The major role of a1ACT is to inhibit cathepsin G, another neutrophil protease that, while less potent than elastase, can also degrade most connective tissue components.

There is increasing evidence that a significant portion of the injury to epithelial cells in the lower respiratory tract in the inflammatory lung disorders are mediated by toxic oxygen radicals released by the inflammatory cells comprising the aleolitis. Probably the most important of these evidients is H_2O_2 since, in the presence of the appropriate catalysts, can be converted into OH. and HC1O-, molecules that are very toxic to normal lung cells. Some protection to the lung parenchymal cells against H_2O_2 may be afforded by antioxidants in the epithelial lining fluid of the lower respiratory tract. Evaluation of epithelial lining fluid from normal individuals demonstrated that it will protect lung parenchymal cells from cytotoxic injury mediated by H_2O_2. This protection is afforded partially through catalase, a major intracellular antioxidant that is likely released into the alveolar epithelial lining fluid (ELF) as part of the turnover of cells in the lower respiratory tract. Additional antioxidant protection in ELF is likely provided by a1AT, ceruloplasmin, and small molecular weight oxidant scavengers.

Under normal circumstances, the alveolar walls provide a "barrier" against a "leak" of plasma components and fluid from the pulmonary capillaries to the alveolar epithelial surface. Using bronchoalveolar lavage and analysis of ELF, alveolar-capillary "leak" can be directly quantified. For example, when 100% oxygen is administered to normal individuals for 18 hours, the concentration of albumin in ELF increases, consistent with the knowledge that hyperoxia for extended periods is toxic to normal lung parenchymal cells. Furthermore, in the chronic inflammatory lung disorders such as ideopathic pulmonary fibrosis and sarcoidosis, the volume of ELF recovered by lavage is increased and the concentration of albumin in ELF is sometimes increased consistent with the knowledge that when there is inflammation in the lower respiratory tract there is usually mild edema of the alveolar walls.

Sarcoidosis is the most common interstitial lung disease of unknown etiology. Studies in the Pulmonary Branch over the last several years have demonstrated

that this disease is associated with large numbers of T-lymphocytes and alveolar macrophages within the alveolar structures. The T-cells are predominantly of the T-helper subtype OKT4+, Leu 3+). Futhermore, in active sarcoid, many also express the cell surface antigens DR, DS and 4F2, surface molecules thought to be associated with "activated" T-cells. Consistent with these observations, the T-cells recovered from the lungs of patients with active sarcoid are spontaneously releasing of mediators that play an important role in the disease process. Thus, central to an understanding of the pathogenesis of this disease is an understanding of what is the mechanism that drives the accumulation and activation of helper T-lymphocytes in lower respiratory tract. In this context, evaluation of lung T-lymphocytes in active pulmonary sarcoidosis has demonstrated that they are spontaneously proliferating at least four to five fold higher than the rate of lung T-cell replication observed in normals.

Furthermore, the lung T-cells are spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor, suggesting that the accumulation of T-cells in the sarcoid lung is mediated, at least in part, but signals within the local milieu. In addition to releasing IL-2, the lung T-cells are spontaneously releasing monocyte chemotactic factor, a mediator that attracts blood monocytes and interferon-gamma, a mediator that activates monomuclear phagocytes, including alveolar macrophages. In this context, it is thought that the lung T-cells play a central role in pulmonary sarcoid by modulating granuloma formation, the accumulation of mononuclear phagocytes into "ball-like" structures. Consistent with the concept that DR+ T-cells are activated, using a fluorescent activated cell sorter to separate sarcoid lung T-cells that are 3+ (helper) DR+ ("activated") from those that are 3+DR- has shown that it is the 3+DR+ cells that are releasing interferon-gamma. Furthermore, using monoclonal antibodies to evaluate the subpopulations of alveolar macrophages in the sarcoid lung, recent studies have shown that a larger proportion than normal are positive with the monoclonal antibodies 63D3, OKM1, MØP-9, MØS-1, 61D3, and MØS-39 (i.e., they are more "monocyte"-like) consistent with the knowledge that the lung T-cells are releasing monocyte chemotactic factor, a mediator that attracts blood monocytes. Interestingly, the alveolar macrophages in the sarcoid lung are also releasing interferon-gamma, and thus, are likely playing a role in "activating" themselves.

Since large numbers of T-lymphocytes are present in the lower respiratory tract in sarcoid patients with active disease and low numbers of T-lymphocytes with patients with inactive disease, a prospective study was conducted to evaluate the natural history of the alveolitis and the alveolitis dependent changes in lung function associated with untreated pulmonary sarcoidosis. Lymphocytes were evaluated by bronchoalveolar lavage and the status of the macrophage populations evaluated by gallium-67 scanning. Two levels of alveolitis were defined: those patients with high intensity alveolitis (lymphocytes representing greater than or equal to 28% of those cells recovered by bronchoalveolar lavage plus a positive thoracic gallium-67 scan) and low intensity alveolitis (patients with either T-lymphocytes in lavage of less than 28% and/or negative gallium-67 scan). Prospective evaluation of patients with sarcoidosis demonstrated the low intensity state is relatively stable whereas the high intensity state can revert to normal in some patients. However, if the high intensity state persists, the patient deteriorates. In contrast, patients with low intensity alveolitis have stable or improving lung functions over a period of time. Recent studies have shown that <u>in vivo</u> treatment of patients with corticosteroids results in a rapid suppression of interleukin-2 release by lung T-cells and a subsequent sup-

pression of lung T-cell proliferation. Furthermore, evaluation of the alveolar macrophage subpopulations with monoclonal antibodies demonstrated a return of the macrophage population from a more "monocyte-like" population to a more macrophage-like" population. In this context, when sarcoid patients with high intensity alveolites are treated with corticosteroids, they had a marked improvement in lung function. As a new approach to the treatment of pulmonary sarcoidosis, in vitro studies have been carried out with cylosporine, a drug used to suppress cell mediated processes during rejection of organ transplants. These studies have shown that when cyclosporine is added to cultures of sarcoid lung T-cells that are spontaneously releasing monocyte chemotactic factor, the release of this mediator by the T-cells is suppressed. Thus, it may be possible to directly attack the activated T-cell in sarcoidosis with specific therapy, particularly in those cases where corticosteroids are unsuccessful or contraindicated.

The etiology of sarcoidosis is unknown. One possibility is that it is caused by an infectious agent such as a retrovirus. In this context, studies were carried out in conjunction with R. Gallo, NCI, to evaluate sarcoid lung T-cells for the presence of HTLV-1, a retrovirus that is one cause of human T-cell lymphoma. Although these studies failed to show evidence of the HTLV-1 genome in the sarcoid lung T-cells, the fact that the activated sarcoid lung T-cell is so similar to a human T-cell infected with HTLV-1 suggests this avenue of research may eventually lead to an identification of the "sarcoid-agent", if it exists.

An alternative hypothesis to the ethology of sarcoidosis is that it is due to an abnormality of alveolar macrophages and/or T-cells such that the normal possess of antigen recognition and activation of the immune system is heightened i.e., that the large numbers of activated helper T-cells accumulate because the response to a normal antigen burden is abnormally heightened. In this context, evaluation of the process of antigen presentation by alveolar macrophages of patients with sarcoid shows that it is heightened approximately 2-fold i.e., for a standard amount of antigen, T-cell proliferation is enhanced twice that observed with normal alveolar macrophages and T-cells.

Berryliosis is a chronic disorder of lung caused by the inhalation of berrylium and that is morphologically indistinct from sarcoidosis. Evaluation of patients with chronic berryliosis has shown that the alveolitis of the disease is also indistinguishable from sarcoid. There are large numbers of T-cells, a markedly increased number of helper (leu3+) T-cells. Furthermore, these lung T-cells are spontaneously proliferating and are releasing IL-2, the T-cell growth factor. However, one major difference between berryliosis and sarcoid is that in berryliosis the lung T-cells proliferate in response to berrylium where as in sarcoid they do not.

Idiopathic pulmonary fibrosis (IPF) is a common interstitial disease of unknown etiology; it is almost invariably fatal with an average course of 4-6 years even with optimal therapy. The abnormalities to the lung in IPF include marked injury to the alveolar structures with progressive fibrosis. The alveolitis of this disease is characterized by large numbers of alveolar macrophages and neutrophils with small numbers of eosinophils. Evaluation of some of the mechanisms of the derangements to the alveolar structures in IPF have demonstrated that the alveolar macrophages, neutrophils and eosinophils all play a role by virtue of their ability to release toxic oxidant radicals. In vitro studies have demonstrated that the inflammatory cells in the lungs of these patients are spontaneously re-

leasing toxic oxygen radicals such as superoxide anion and H_2O_2 at a rate greater than that of normal. Furthermore, the alveolar macrophages of these patients are spontaneously cytotoxic for lung parenchymal cells. One mechanism of the macrophage activation of IPF is by immune complexes probably formed within the lung. Consistent with the concept that neutrophils play a role in the alveolites of IPF, myleoperoxidase (MPO), a neutrophil product, is found in the alveolar ELF of these patients. Furthermore, since MPO can catalyze the formation of the hypohalide anion from H_2O_2, and since inflammatory cells from the lungs of IPF patients spontaneously release H_2O_2, it is likely that MPO augments the injury to the alveolar epithelial cells of these patients. Consistent with this concept, when ELF recovered from patients with active IPF are added to an in vitro test system in which H_2O_2 is generated in the presence of lung epithelial cells, there is augmented cytotoxicity to the cells.

Pulmonary fibrosis is the accumulation of fibroblasts and fibroblast products (particularly type I collagen) in areas of injury and derangement of the normal alveolar structures. Evaluation of lung biopsies of patients with interstitial lung disease as well as lungs of monkeys exposed to paraquat (a herbacide that, if it gains access to the body, can cause severe derangement to the alveolar structures) demonstrates that two forms of fibrosis are common: (1) interstitial fibrosis which expands the alveolar walls; and (2) intraalveolar fibrosis which occupies space within the alveolar airspaces. Whereas both are associated with an accumulation of fibroblasts at the site of fibrosis, the intraalveolar fibrosis also requires a break of the epithelial basement membrane, a process that seems to occur in situations in which the alveotitis is intense, particularly when neutrophils are involved.

Because pulmonary fibrosis is invariably associated with an increase in fibroblast numbers, the concept has developed that fibrosis of the alveolar structures occurs when, in regions of injury and derangement of the alveolar walls, there is recruitment, attachment and proliferation of fibroblasts. Current concepts of cell replication suggests that exogenous mediators are capable of signalling resting fibroblasts to multiply. These mediators can conveniently be divided into "competence factors" (growth factors that act early in G1) and "progression factors" (factors that act later than G1 and allow the cell to proceed to DNA synthesis). The human alveolar macrophage is capable of releasing a competence factor as well as a progression factor for fibroblasts. Furthermore, human alveolar macrophages obtained from patients with sarcoidosis, asbestos exposure, coal dust exposure, and oxygen toxicity, are releasing larger amounts of these mediators than alveolar macrophages of normal individuals. The competence factor is fibronectin, a 440,000 dalton polypeptide that acts early in the G1 phase of the cell cycle to signal the cells to respond to the progression factor, alveolar macrophage derived growth factor, a 16,000 dalton protein that is different from all other described growth factors. Fibronectin likely has other roles in the fibrotic lung diseases; it is a chemoattractant for fibroblasts and will attach fibroblasts to the connective tissue matrix. Furthermore, fibronectin can attach fibroblasts to immune complexes by virtue of its ability to attach fibroblasts to C1q, one of the early components of the complement cascade. In this context, if antibodies bind to tissue components, C1q may attach to the antibody and fibroblasts attach to C1q through fibronectin, i.e., the immune complex forms the nidus for the localization of fibroblasts and hence the nidus for the development of fibrosis in the local environment.

Although glucorticoids are the standard therapy for the fibrotic lung diseases, recent studies have shown that when patients with IPF are treated with corticosteroids, their alveolar macrophages continue to produce fibronectin and alveolar macrophage derived growth factor, dispite the fact that their alveolar macrophages appear to have receptors for corticosteroids similar to that of normals. In this context, the concept has developed that, to successfully treat the fibrotic disorders, it will be necessary to use agents that successfully suppress the alveolar macrophages. In vitro studies have shown that a prototype of such an agent is colchicine, a drug that when added to cultures of alveolar macrophages of patients with IPF, suppresses the release of fibronectin and alveolar macrophage derived growth factor.

The most important "model" for understanding the pathogenesis of destructive lung disease is alpha 1-antitrypsin (alAT deficiency, a hereditary disorder characterized by a marked reduction in serum levels of alAT, the liver produced antiprotease that normally provides the bulk of anti-neutrophil elastase defense to the lower respiratory tract. alAT is a glycoprotein that consists of 394 amino acids and 3 carbohydrate side chains. It has a high affinity for neutrophil elastase (association rate constant 5x10 7 M-1 sec-1) through an active site in the alAT site centered at methionine 358. Both parental genes of alAT are expressed in a codominant fashion. Conventionally, alpha 1-AT polymorphisms are defined at the protein level by isoelectric focusing of serum at pH 4.5-5.0. Most caucasians of northern European descent have M-type alAT; individuals who have the alAT phenotype MM have serum levels of 150-250 mg/dl, levels thought to be sufficient to provide adequate protection to the alveolar structures. In contrast, individuals homozygous for the Z type alAT (glutanic acid 342 --- lysine 342) have serum levels of 10 to 50 mg/dl and almost invariably develop emphysema in the fourth to fifth decade of life.

In collaberation with scientists at Transgene, Strasbourg, France, the Pulmonary Branch has begun a detailed analysis of the alAT gene structure and expression. The alAT gene is known to be spread over approximately 11 kb of DNA on chromosome 14. It contains 5 exons and 4 introns; the sequences actually coding for alAT are found over 1.4 kb of DNA in exons II to V. Using a cDNA probe complementary to the human alAT gene exons, DNA from individuals with Ml type alpha 1-AT (the most common M-type representing approximately 60-70% of all individuals) was compared to DNA of the Z type alpha 1-AT for the restriction fragments produced with a variety of restriction endonucleases. With the enzyme TaqI, a restriction length polymorphism has been found associated with approximately 11% of the Ml genes but which is absent in all of the Z genes. Mapping techniques have localized this polymorphism site approximately 1.4 kb 3' to exon V of the alpha 1-AT. While it is not clear whether this particular polymorphism has clinical relevance, it demonstrates that the alAT gene is likely more polymorphic than that recognized at the protein level by isoelectric focusing and that such polymorphisms may be unequally distributed among individuals with alAT types thought to be "normal". In this context, analysis of alAT at the DNA level may provide new markers by which to evaluate patients for the risk of development of emphysema.

The human alAT cDNA has been inserted into an expressing plasmid by Transgene and the host E.coli induced to produce alAT molecules. One of these molecules, TG1 (E.coli), has been evaluated in detail by the Pulmonay Branch. It has a molecular weight of 45,000, contains (N-terminal to C-terminal) 17 amino acids of plasmid origin and 393 of the 394 amino acids of normal alpha 1-AT, but has no carbohydrates. Comparison of T61(E.coli) and normal human plasma alAT demon-

strates that the association rate constant of the two are equal i.e., the E.coli produced material is as good an inhibitor of neutrophil elastase as is the plasma alA T.

The human alAT cDNA has also been used to evaluate alAT gene expression at the mRNA level. Although the liver is the major site of synthesis of alAT, it is also known to be produced by mononuclear phagocytes. Using human alveolar macrophages as a model for a cell that produces alAT, analysis with Northern and dot hybridization techniques has shown that alveolar macrophages from individuals with M type alAT and Z type alAT have approximately equivalent amounts of alAT mRNA. Interestingly, while much less (per cell) than liver, the alAT mRNA levels in alveolar macrophages are much higher than that in blood monocytes, suggesting there is an alteration in alAT gene expression during the maturation of monocytes to macrophages.

Since alAT deficiency is a disorder characterized by a marked reduction in the levels of a serum protein, one approach to the therapy of this disorder is to replace the missing protein by intermittant infusions of alAT. A few years previously, the Pulmonary Branch demonstrated that if 4 gms of alAT were infused intravenously once weekly to these individuals, the alAT levels could be maintained at greater than or equal to 80 mg/dl, a level thought to be protective against the development of emphysema. In collabration with Cutter Laboratories, Palo Alto, California, the Pulmonary Branch has begun an evaluation of alAT replacement therapy in a small group of individuals. Preliminary studies suggest the material is safe and that infusions in the range of 4 gm once weekly should be sufficient to maintain alAT levels above those thought to be protective.

Recent studies have demonstrated that cigarette smoking in individuals with normal levels of alAT may be associated with inactivation of the alpha 1-AT in the lungs. Since cigarette smokers also may accumualte neutrophils (a source of elastase) in the lungs, cigarette smokers may have an imbalance of elastase and antielastase in the lower respiratory tract in favor of the elastase. The neutrophil accumulation occurs because particulates in cigarette smoke induce human alveolar macrophages to release a low molecular weight chemotactic factor that selectively attracts neutrophils. Another role for alveolar macrophages in the destruction of alveolar walls associated with cigarette smoking is the stimulation of the alveolar macrophages by cigarette smoke to release toxic oxygen radicals such as superoxide anion and hydrogen peroxide. Furthermore, alveolar macrophages from cigarette smokers are spontaneously cytotoxic for normal lung parenchymal cells. While the mechanism of how cigarette smoking leads to emphysema is likely very complex, these observations suggest that the proteases-antiprotease and oxidant-antioxidant balance in the lower respiratory tract play a major role in determining which individuals are susceptable to cigarette smoking induced disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z-01-HL-02405-11 PB

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interstitial Lung Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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LAB/BRANCH
Pulmonary Branch

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
31	24	7

CHECK APPROPRIATE BOX(ES)
- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interstitial lung disorders represent 15 to 20 percent of all diseases of the alveolar structures; in most cases these disorders cause significant disability and many are fatal. Studies of the natural history, etiology, pathogenesis and therapy of these disorders have made major inroads in the understanding of these diseases. Most importantly, is the development of the concept of the inflammatory and immune effector cells which are critical determinants in the pathogenic process. Evaluation of the alveolitis of these patients has led to significant insights into the processes by which the alveolar structures are injured and repaired. As part of these studies, insights have been gained into the role of the alveolar macrophage, T-lymphocyte, neutrophil, and eosinophils as well as how fibroblasts are recruited, anchored and stimulated to replicate. Particular emphasis has been placed on the disorders, idiopathic pulmonary fibrosis and sarcoidosis, common interstitial lung disorders of unknown etiology. Advancements have been made relating to the pathogensis of these disorders and new approaches taken directed at suppressing the inflammation that causes the lung derangement and eventual respiratory insufficiency associated with these diseases.

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Project Description:
The interstitial lung disorders are a group of heterogeneous, chronic diseases of the alveolar structures characterized by an alveolitis, changes in alveolar epithelial, endothelial, and mesenchymal cells, and alterations in the alveolar connective tissue matrix referred to as "fibrosis". The interstitial disorders of known etiology represent 35% of all interstitial disorders and include the inhalational disorders (e.g., the pneumoconioses, hypersensitivity pneumonitis) as well as the disorders resulting from radiation, drugs and poisons. There are many interstitial lung disorders of unknown etiology but some of the most commonly encountered are sarcoidosis and idiopathic pulmonary fibrosis (IPF). The current concepts of the pathogenesis of all of the interstitial diseases suggest that it is the specific inflammatory and immune processes comprising the alveolitis that modulate the changes to the parenchymal cells and connective tissue matrix that define the physiologic abnormalities that characterize each disorder.

The technique of bronchoalveolar lavage permits direct access to the epithelial surface of the lower respiratory tract where inflammatory cells and relevant molecules in the epithelial lining fluid can be easily and repetitively sampled in normals and patients with interstitial disorders. To accomplish bronchoalveolar lavage, local anesthesia is administered to the upper respiratory tract and a fiberoptic bronchoscope is gently wedged into a distal bronchus. Sterile saline, usually in 5, 20 ml aliquots in 1 to 3 sites, is infused into the bronchoscope and then suctioned back, thus sampling the epithelial lining fluid of the lower respiratory tract.

In the normal lung, the majority of the inflammatory cells in the alveolar structures are alveolar macrophages, cells derived from the mononuclear phagocyte system. The remaining inflammatory cells are lymphocytes. Recent studies have demonstrated that the usual techniques of utilizing a cytocentrifuge to prepare cells recovered by lavage for analysis underestimated the actual proportion of lymphocytes present. With a millipore filtering method, however, absolute quantitation of the cell type can be accomplished. With this approach, studies of normal individuals reveals that, on the average, 83% of cells are alveolar macrophages and 17% are lympocytes.

The normal nonsmoking human has small numbers of alveolar macrophages per alveolus (approximately 50 to 70). Alveolar macrophages are thought to be the "directors" of inflammatory processes in the lower respiratory tract. In addition to being phagocytic, these cells can direct lymphocytes to proliferate, suppress immune processes, and recruit inflammatory cells such as neutrophils. Using monoclonal antibodies directed toward cell surface antigens of cells of the mononuclear phagocyte series it has been possible to demonstrate that the population of alveolar macrophages can be categorized into subpopulations depending on their cell surface characteristics. For example, anti-DR (an antibody directed against a non-polymorphic HLA-DR epitope) is positive in or greater than 90% of normal alveolar macrophages, while ODT9 (an antibody directed against the transferrin recptor) is positive on approximately 30% of alveolar macrophages, and OKM1 (an antibody that recognized the C3Bi receptor) is positive in 15 to 35% of normal alveolar macrophages. At least part of these subpopulation differences appears to be related to the process of maturation of mononuclear phagocytes. It is known that alveolar macrophages are derived from

blood monocytes. As a model of monocyte to alveolar macrophage differentiation, blood monocytes are placed in culture and evaluated for cell surface characteristics over time. Whereas blood monocytes are OKT9- (i.e., they do not express the transferrin receptor), as they "mature" in culture, the transferrin receptor is expressed. Likewise, wheras approximately 90% normal blood monocytes express the cell surface antigens 63D3, MØP-9, mØS-1 and MØS-39, in culture they rapidly lose these cell surface characteristics, consistent with the findings that only 5 to 40% of normal alveolar macrophages express the same antigens.

Normal functioning of the immune system requires communication between the mononuclear phagocyte system and T-lymphocytes. One means by which this occurs is through a mediator, interleukin-1, a 16,000 dalton protein produced by mononuclear phagocytes that plays an important role in the early steps of T-cell proliferation in response to antigens. Recent studies have demonstrated that human alveolar macrophages release far less interleukin-1 (approximately 20% with a standard stimulus) than do human blood monocytes. Since the lung is heavily exposed to antigens, the fact that a critical mediator may be rate limiting may be one mechanism by which antigenic responses are controlled in the lower respiratory tract. Consistent with this observation, normal human alveolar macrophages "present" antigens to autologous blood or lung T-lymphocytes in a manner that results in approximately one-quarter of the T-cell proliferation as do autoloqous blood monocytes. Thus, if a population of normal blood monocytes and T-lymphocytes were exposed to the same quantity of antigen as normal alveolar macrophages and T-lymphocytes, the T-cells exposed to the monocytes would proliferate to a much greater extent.

Classical concepts of how mononuclear phagocytes accumulate in areas of inflammation suggest that all are recruited from blood monocytes that are called to the the site of inflammation. However, recent studies demonstrated that human alveolar macrophages are capable of replicating and they do so in many inflammatory lung disorders. Thus, the increase in macrophage numbers found in these diseases probably represent, in part, local proliferation together with recruitment from blood monocytes. While the mechanisms inducing this macrophase replication are not known, growth factors in the local inflammatory milieu are likely responsible. In this regard, the fact that alveolar macrophages can proliferate is consistent with the knowledge that transferrin is required for cell proliferation and that a significant proportion of normal alveolar macrophages express the transferin receptor.

The normal human lung contains approximately 9 to 12 lymphocytes per alveolus. Using monoclonal antibodies directed toward cell surface antigens, studies have demonstrated that approximately 90% are T-lymphocytes (OKT3+, Leu 4+) and 2% are B-lymphocytes (B1+). Of the T-cells, approximately 65% have surface antigens characteristic of "helper" cells (OKT4+, Leu 3+) while 30-35% are characteristic of "suppressor/cytotoxic" cells (OKT8+, Leu 2+). Very few of the 3+ or 2+ normally express surface antigens suggesting they are activated (e.g., DR, DS, and 4F2 antigens). Consistent with this observation, <u>in vitro</u> evaluation of normal lung T-cells shows that they are not spontaneously releasing mediators such as interferon-gamma, interleukin-2, or monocyte chemotactic factor. Although 2% of the lympocytes on the epithelial surface of the lower respiratory tract are B-cells, evaluation of these cells demonstrates that, unlike normal blood B-cells, they are not spontaneously releasing immunoglobulins. The rea-

son for this is not clear, but likely relates to the fact that the normal milieu of the alveolar epithelial surface suppresses inflammatory processes. Consistent with this concept, morphologic studies of the lymphocytes recovered from the normal lower respiratory tract show that approximately 10% are "large granular lymphocytes", characteristics of natural-killer cells (cells of the lymphocyte series that are spontaneously cytotoxic for tumor cells). Interestingly, however, there is very little natural killer cell activity among the lymphocyte populations of the lung. This lack of lung natural killer cell activity may be due, in part, to the ability of human alveolar macrophages and alveolar epithelial living fluid to suppress natural killer cells. However, when interleukin-2, the T-cell growth factor, is added to a population of lung lymphocytes, natural killer activity is expressed. As a corollary to this observation, lung lymphocytes in active sarcoidosis do have natural killer cell activity, a finding consistent with the knowledge that lung T-cells in active are sarcoid are spontaneously releasing interleukin-2. Furthermore, in vitro studies with natural killer cells have demonstrated that chrysotile asbestosis suppress natural killer activity, an observation consistent with the knowledge that long term exposure to high levels of chrysotile asbestos increases the risk of lung cancer in cigarette smokers.

There is increasing evidence that a significant portion of the injury to epithelial cells in the lower respiratory tract in the inflammatory lung disorders are mediated by toxic oxygen radicals released by the inflammatory cells comprising the aleolitis. Probably the most important of these evidients is H_2O_2 since, in the presence of the appropriate catalysts, can be converted into OH. and $HClO^-$, molecules that are very toxic to normal lung cells. Some protection to the lung parenchymal cells against H_2O_2 may be afforded by antioxidants in the epithelial lining fluid of the lower respiratory tract. Evaluation of epithelial lining fluid from normal individuals demonstrated that it will protect lung parenchymal cells from cytotoxic injury mediated by H_2O_2. This protection is afforded partially through catalase, a major intracellular antioxidant that is likely released into the alveolar epithelial lining fluid (ELF) as part of the turnover of cells in the lower respiratory tract. Additional antioxidant protection in ELF is likely provided by alAT, ceruloplasmin, and small molecular weight oxidant scavengers.

Under normal circumstances, the alveolar walls provide a "barrier" against a "leak" of plasma components and fluid from the pulmonary capillaries to the alveolar epithelial surface. Using bronchoalveolar lavage and analysis of ELF, alveolar-capillary "leak" can be directly quantified. For example, when 100% oxygen is administered to normal individuals for 18 hours, the concentration of albumin in ELF increases, consistent with the knowledge that hyperoxia for extended periods is toxic to normal lung parenchymal cells. Furthermore, in the chronic inflammatory lung disorders such as ideopathic pulmonary fibrosis and sarcoidosis, the volume of ELF recovered by lavage is increased and the concentration of albumin in ELF is sometimes increased consistent with the knowledge that when there is inflammation in the lower respiratory tract there is usually mild edema of the alveolar walls.

Sarcoidosis is the most common interstitial lung disease of unknown etiology. Studies in the Pulmonary Branch over the last several years have demonstrated that this disease is associated with large numbers of T-lymphocytes and alveolar

macrophages within the alveolar structures. The T-cells are predominantly of the T-helper subtype OKT4+, Leu 3+). Futhermore, in active sarcoid, many also express the cell surface antigend DR, DS and 4F2, surface molecules thought to be associated with "activated" T-cells. Consistent with these observations, the T-cells recovered from the lungs of patients with active sarcoid are spontaneously releasing of mediators that play an important role in the disease process. Thus, central to an understanding of the pathogenesis of this disease is an understanding of what is the mechanism that drives the accumulation and activation of helper T-lymphocytes in lower respiratory tract. In this context, evaluation of lung T-lymphocytes in active pulmonary sarcoidosis has demonstrated that they are spontaneously proliferating at least four to five fold higher than the rate of lung T-cell replication observed in normals.

Furthermore, the lung T-cells are spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor, suggesting that the accumulation of T-cells in the sarcoid lung is mediated, at least in part, but signals within the local milieu. In addition to releasing IL-2, the lung T-cells are spontaneously releasing monocyte chemotactic factor, a mediator that attracts blood monocytes and interferon-gamma, a mediator that activates monomuclear phagocytes, including alveolar macrophages. In this context, it is thought that the lung T-cells play a central role in pulmonary sarcoid by modulating granuloma formation, the accumulation of mononuclear phagocytes into "ball-like" structures. Consistent with the concept that DR+ T-cells are activated, using a fluorescent activated cell sorter to separate sarcoid lung T-cells that are 3+ (helper) DR+ ("activated") from those that are 3+DR- has shown that it is the 3+DR+ cells that are releasing interferon-gamma. Furthermore, using monoclonal antibodies to evaluate the subpopulations of alveolar macrophages in the sarcoid lung, recent studies have shown that a larger proportion than normal are positive with the monoclonal antibodies 63D3, OKM1, MØP-9, MØS-1, 61D3, and MØS-39 (i.e., they are more "monocyte"-like) consistent with the knowledge that the lung T-cells are releasing monocyte chemotactic factor, a mediator that attracts blood monocytes. Interestingly, the alveolar macrophages in the sarcoid lung are also releasing interferon-gamma, and thus, are likely playing a role in "activating" themselves.

Since large numbers of T-lymphocytes are present in the lower respiratory tract in sarcoid patients with active disease and low numbers of T-lymphocytes with patients with inactive disease, a prospective study was conducted to evaluate the natural history of the alveolitis and the alveolitis dependent changes in lung function associated with untreated pulmonary sarcoidosis. Lymphocytes were evaluated by bronchoalveolar lavage and the status of the macrophage populations evaluated by gallium-67 scanning. Two levels of alveolitis were defined: those patients with high intensity alveolitis (lymphocytes representing greater than or equal to 28% of those cells recovered by bronchoalveolar lavage plus a positive thoracic gallium-67 scan) and low intensity alveolitis (patients with either T-lymphocytes in lavage of less than 28% and/or negative gallium-67 scan). Prospective evaluation of patients with sarcoidosis demonstrated the low intensity state is relatively stable whereas the high intensity state can revert to normal in some patients. However, if the high intensity state persists, the patient deteriorates. In contrast, patients with low intensity alveolitis have stable or improving lung functions over a period of time. Recent studies have

shown that in vivo treatment of patients with corticosteroids results in a rapid suppression of interleukin-2 release by lung T-cells and a subsequent suppression of lung T-cell proliferation. Furthermore, evaluation of the alveolar macrophage subpopulations with monoclonal antibodies demonstrated a return of the macrophage population from a more "monocyte-like" population to a more macrophage-like" population. In this context, when sarcoid patients with high intensity alveolites are treated with corticosteroids, they had a marked improvement in lung function. As a new approach to the treatment of pulmonary sarcoidosis, in vitro studies have been carried out with cylosporine, a drug used to suppress cell mediated processes during rejection of organ transplants. These studies have shown that when cyclosporine is added to cultures of sarcoid lung T-cells that are spontaneously releasing monocyte chemotactic factor, the release of this mediator by the T-cells is suppressed. Thus, it may be possible to directly attack the activated T-cell in sarcoidosis with specific therapy, particularly in those cases where corticosteroids are unsuccessful or contraindicated.

The etiology of sarcoidosis is unknown. One possibility is that it is caused by an infectious agent such as a retrovirus. In this context, studies were carried out in conjunction with R. Gallo, NCI, to evaluate sarcoid lung T-cells for the presence of HTLV-1, a retrovirus that is one cause of human T-cell lymphoma. Although these studies failed to show evidence of the HTLV-1 genome in the sarcoid lung T-cells, the fact that the activated sarcoid lung T-cell is so similar to a human T-cell infected with HTLV-1 suggests this avenue of research may eventually lead to an identification of the "sarcoid-agent", if it exists.

An alternative hypothesis to the ethology of sarcoidosis is that it is due to an abnormality of alveolar macrophages and/or T-cells such that the normal possess of antigen recognition and activation of the immune system is heightened i.e., that the large numbers of activated helper T-cells accumulate because the response to a normal antigen burden is abnormally heightened. In this context, evaluation of the process of antigen presentation by alveolar macrophages of patients with sarcoid shows that it is heightened approximately 2-fold i.e., for a standard amount of antigen, T-cell proliferation is enhanced twice that observed with normal alveolar macrophages and T-cells.

Berryliosis is a chronic disorder of lung caused by the inhalation of berrylium and that is morphologically indistinct from sarcoidosis. Evaluation of patients with chronic berryliosis has shown that the alveolitis of the disease is also indistinguishable from sarcoid. There are large numbers of T-cells, a markedly increased number of helper (leu3+) T-cells. Furthermore, these lung T-cells are spontaneously proliferating and are releasing IL-2, the T-cell growth factor. However, one major difference between berryliosis and sarcoid is that in berryliosis the lung T-cells proliferate in response to berrylium where as in sarcoid they do not.

Idiopathic pulmonary fibrosis (IPF) is a common interstitial disease of unknown etiology; it is almost invariably fatal with an average course of 4-6 years even with optimal therapy. The abnormalities to the lung in IPF include marked injury to the alveolar structures with progressive fibrosis. The alveolitis of this disease is characterized by large numbers of alveolar macrophages and neutrophils with small numbers of eosinophils. Evaluation of some of the mechanisms of the derangements to the alveolar structures in IPF have demonstrated that the alveo-

lar macrophages, neutrophils and eosinophils all play a role by virtue of their ability to release toxic oxidant radicals. In vitro studies have demonstrated that the inflammatory cells in the lungs of these patients are spontaneously releasing toxic oxygen radicals such as superoxide anion and H2O2 at a rate greater than that of normal. Furthermore, the alveolar macrophages of these patients are spontaneously cytotoxic for lung parenchymal cells. One mechanism of the macrophage activation of IPF is by immune complexes probably formed within the lung. Consistent with the concept that neutrophils play a role in the alveolites of IPF, myleoperoxidase (MPO), a neutrophil product, is found in the alveolar ELF of these patients. Furthermore, since MPO can catalyze the formation of the hypohalide anion from H2O2, and since inflammatory cells from the lungs of IPF patients spontaneously release H2O2, it is likely that MPO augments the injury to the alveolar epithelial cells of these patients. Consistent with this concept, when ELF recovered from patients with active IPF are added to an in vitro test system in which H2O2 is generated in the presence of lung epithelial cells, there is augmented cytotoxicity to the cells.

Pulmonary fibrosis is the accumulation of fibroblasts and fibroblast products (particularly type I eollagen) in areas of injury and derangement of the normal alveolar structures. Evaluation of lung biopsies of patients with interstitial lung disease as well as lungs of monkeys exposed to paraquat (a herbacide that, if it gains access to the body, can cause severe derangement to the alveolar structures) demonstrates that two forms of fibrosis are common: (1) interstitial fibrosis which expands the alveolar walls; and (2) intraalveolar fibrosis which occupies space within the alveolar airspaces. Whereas both are associated with an accumulation of fibroblasts at the site of fibrosis, the intraalveolar fibrosis also requires a break of the epithelial basement membrane, a process that seems to occur in situations in which the alveotitis is intense, particularly when neutrophils are involved.

Because pulmonary fibrosis is invariably associated with an increase in fibroblast numbers, the concept has developed that fibrosis of the alveolar structures occurs when, in regions of injury and derangement of the alveolar walls, there is recruitment, attachment and proliferation of fibroblasts. Current concepts of cell replication suggests that exogenous mediators are capable of signalling resting fibroblasts to multiply. These mediators can conveniently be divided into "competence factors" (growth factors that act early in G1) and "progression factors" (factors that act later than G1 and allow the cell to proceed to DNA synthesis). The human alveolar macrophage is capable of releasing a competence factor as well as a progression factor for fibroblasts. Furthermore, human alveolar macrophages obtained from patients with sarcoidosis, asbestos exposure, coal dust exposure, and oxygen toxicity, are releasing larger amounts of these mediators than alveolar macrophages of normal individuals. The competence factor is fibronectin, a 440,000 dalton polypeptide that acts early in the G1 phase of the cell cycle to signal the cells to respond to the progression factor, alveolar macrophage derived growth factor, a 16,000 dalton protein that is different from all other described growth factors. Fibronectin likely has other roles in the fibrotic lung diseases; it is a chemoattractant for fibroblasts and will attach fibroblasts to the connective tissue matrix. Furthermore, fibronectin can attach fibroblasts to immune complexes by virtue of its ability to attach fibroblasts to C1q, one of the early components of the complement cascade. In this context, if antibodies bind to tissue components, C1q may attach

to the antibody and fibroblasts attach to Clq through fibronectin, i.e., the immune complex forms the nidus for the localization of fibroblasts and hence the nidus for the development of fibrosis in the local environment.

Although glucorticoids are the standard therapy for the fibrotic lung diseases, recent studies have shown that when patients with IPF are treated with corticosteroids, their alveolar macrophages continue to produce fibronectin and alveolar macrophage derived growth factor, dispite the fact that their alveolar macrophages appear to have receptors for corticosteroids similar to that of normals. In this context, the concept has developed that, to successfully treat the fibrotic disorders, it will be necessary to use agents that successfully suppress the alveolar macrophages. In vitro studies have shown that a prototype of such an agent is colchicine, a drug that when added to cultures of alveolar macrophages of patients with IPF, suppresses the release of fibronectin and alveolar macrophage derived growth factor.

Significance to Biomedical Research and the Program of the Institute: The interstitial lung disorders and destructive lung disorders are almost uniformly fatal and affect a significant proportion of the population. There has been little information on the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders. By combining studies of patients with these disorders with our basic research program concerning basic immunology as well as evaluation of the control of synthesis and degradation of the extracellular matrix, we expect to make major inroads into understanding and treating these disorders.

Proposed Course: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to the study of biologic materials obtained from the lungs of patients. Of particular importance are the studies relating to the inflammatory and immune processes in the lower respiratory tract as evaluated by bronchoalveolar lavage. As the laboratory studies relating to the therapy of the interstitial lung disorders become promising, they will be studied in patients with disease.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z-01-HL-02407-10 PB

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
Destructive Lung Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

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LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
10	9	2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

The destructive lung disorders are diseases in which there is desolution and loss of alveolar structures. The primary mechanism responsible for this is an imbalance of proteases and antiproteases toward proteases, particularly neutrophil elastase. Studies of cigarette smokers have demonstrated that they have neutrophils in their lungs, and the major antielastase of the lower respiratory tract, alpha-1 antitrypsin, is functionally impotent. The alveolar macrophages play a major role in the pathogenesis of cigarette smoking induced emphysema by virtue of their ability to mediate lung injury and recruit neutrophils. Molecular biologic approaches have been used to understand the structure and expression of the alpha-1 antitrypsin gene in order to give insights as to the control of the macromolecule directly responsible for protection of the alveolar structures against elastase.

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Other Investigators (Cont.)

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Project Description:
The destructive lung disorders are chronic diseases of the lower respiratory tract characterized by the loss of the alveolar structures. The current concepts of the pathogenesis of these disorders are defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in normal lung, proteases (e.g., neutrophil elastase, an enzyme that can destroy most lung connective tissue components) released by inflammatory cells within the alveolar structures, are balanced by antiproteases (e.g., alpha 1-antitrypsis, a circulating antielastase that diffuses into the lung). In the destructive lung diseases, there is increasing evidence that there is an imbalance such that a chronic alveolitis presents an elastase burden that overpowers an insufficient antielastase protective screen, thus causing connective tissue destruction and loss of the alveolar structures.

In addition to inflammatory cells, the technique of bronchoalveolar lavage permits the sampling of the ephthelial lining fluid (ELF) and thus allows analysis if the various macromolecules that play a role in mediating inflammatory and immune processes as well as defending the lung. Some of these molecules include immunoglobulins, complement components, arachadonic acid metabolites (e.g., prostaglandins, leukotrienes), surface active material, antiproteases and antioxidants. It is difficult to quantify the macromolecules in this fluid in absolute terms because the fluid returned is mixed with the large amounts of saline (usually in a 100 to 1 ratio) used for the lavage procedure. One approach is to compare the amount of each component to albumin, a method that permits comparison to values in plasma. Recent studies have demonstrated that by quantifying the amount of urea present in the returned lavage fluid and comparing that to a concentration of urea in the plasma, an absolute estimate of epithelial lining fluid (the "apparent urea volume of epithelial lining fluid") can be determined. It is now possible, therefore, to quantify in absolute terms the amounts of macromolecules in the epithelial lining fluid and to compare these in disease states.

Proteases, enzymes capable of destroying proteins, particularly those comprising the connective tissue matrix that provide the supporting network of the alveolar walls, are released in the lower respiratory tract by inflammatory cells. The two major antiproteases of human alveolar ELF are alpha 1-antitrypsin (a1 AT) and alpha 1-antichymotrypsis (a1ACT). Both are produced primarily in the liver; they are secreted into the blood where, because of their molecule weight (50-60,000 daltons) are capable of diffusing through the alveolar walls. The concentration of a1AT in normal plasma is 150-250 mg/dl and that of a1ACT is 35-60 mg/dl; in ELF the concentrations of both are approximately 10% of that in plasma. a1AT is the major antiprotease capable of defending against neutrophil elastase, an omniverous protease capable of degrading all connective tissue components. The major role of a1ACT is to inhibit cathepsin G, another neutrophil protease that, while less potent than elastase, can also degrade most connective tissue components.

The most important "model" for understanding the pathogenesis of destructive lung disease is alpha 1-antitrypsin (a1AT deficiency, a hereditary disorder characterized by a marked reduction in serum levels of a1AT, the liver produced antiprotease that normally provides the bulk of anti-neutrophil elastase defense to the lower respiratory tract. a1AT is a glycoprotein that consists of 394 amino acids

and 3 carbohydrate side chains. It has a high affinity for neutrophil elastase (association rate constant 5x10 7 M-1 sec-1) through an active site in the alAT site centered at methionine 358. Both parental genes of alAT are expressed in a codominant fashion. Conventionally, alpha 1-AT polymorphisms are defined at the protein level by isoelectric focusing of serum at pH 4.5-5.0. Most caucasians of northern European descent have M-type alAT; individuals who have the alAT phenotype MM have serum levels of 150-250 mg/dl, levels thought to be sufficient to provide adequate protection to the alveolar structures. In contrast, individuals homozygous for the Z type alAT (glutanic acid 342 --- lysine 342) have serum levels of 10 to 50 mg/dl and almost invariably develop emphysema in the fourth to fifth decade of life.

In collaberation with scientists at Transgene, Strasbourg, France, the Pulmonary Branch has begun a detailed analysis of the alAT gene structure and expression. The alAT gene is known to be spread over approximately 11 kb of DNA on chromosome 14. It contains 5 exons and 4 introns; the sequences actually coding for alAT are found over 1.4 kb of DNA in exons II to V. Using a cDNA probe complementary to the human alAT gene exons, DNA from individuals with Ml type alpha 1-AT (the most common M-type representing approximately 60-70% of all individuals) was compared to DNA of the Z type alpha 1-AT for the restriction fragments produced with a variety of restriction endonucleases. With the enzyme TaqI, a restriction length polymorphism has been found associated with approximately 11% of the Ml genes but which is absent in all of the Z genes. Mapping techniques have localized this polymorphism site approximately 1.4 kb 3' to exon V of the alpha 1-AT. While it is not clear whether this particular polymorphism has clinical relevance, it demonstrates that the alAT gene is likely more polymorphic than that recognized at the protein level by isoelectric focusing and that such polymorphisms may be unequally distributed among individuals with alAT types thought to be "normal". In this context, analysis of alAT at the DNA level may provide new markers by which to evaluate patients for the risk of development of emphysema.

The human alAT cDNA has been inserted into an expressing plasmid by Transgene and the host E.coli induced to produce alAT molecules. One of these molecules, TG1 (E.coli), has been evaluated in detail by the Pulmonay Branch. It has a molecular weight of 45,000, contains (N-terminal to C-terminal) 17 amino acids of plasmid origin and 393 of the 394 amino acids of normal alpha 1-AT, but has no carbohydrates. Comparison of T61(E.coli) and normal human plasma alAT demonstrates that the association rate constant of the two are equal i.e., the E.coli produced material is as good an inhibitor of neutrophil elastase as is the plasma alA T.

The human alAT cDNA has also been used to evaluate alAT gene expression at the mRNA level. Although the liver is the major site of synthesis of alAT, it is also known to be produced by mononuclear phagocytes. Using human alveolar macrophages as a model for a cell that produces alAT, analysis with Northern and dot hybridization techniques has shown that alveolar macrophages from individuals with M type alAT and Z type alAT have approximately equivalent amounts of alAT mRNA. Interestingly, while much less (per cell) than liver, the alAT mRNA levels in alveolar macrophages are much higher than that in blood monocytes, suggesting there is an alteration in alAT gene expression during the maturation of monocytes to macrophages.

Since alAT deficiency is a disorder characterized by a marked reduction in the levels of a serum protein, one approach to the therapy of this disorder is to replace the missing protein by intermittant infusions of alAT. A few years previously, the Pulmonary Branch demonstrated that if 4 gms of alAT were infused intravenously once weekly to these individuals, the alAT levels could be maintained at greater than or equal to 80 mg/dl, a level thought to be protective against the development of emphysema. In collabration with Cutter Laboratories, Palo Alto, California, the Pulmonary Branch has begun an evaluation of alAT replacement therapy in a small group of individuals. Preliminary studies suggest the material is safe and that infusions in the range of 4 gm once weekly should be sufficient to maintain alAT levels above those thought to be protective.

Recent studies have demonstrated that cigarette smoking in individuals with normal levels of alAT may be associated with inactivation of the alpha 1-AT in the lungs. Since cigarette smokers also may accumualte neutrophils (a source of elastase) in the lungs, cigarette smokers may have an imbalance of elastase and antielastase in the lower respiratory tract in favor of the elastase. The neutrophil accumulation occurs because particulates in cigarette smoke induce human alveolar macrophages to release a low molecular weight chemotactic factor that selectively attracts neutrophils. Another role for alveolar macrophages in the destruction of alveolar walls associated with cigarette smoking is the stimulation of the alveolar macrophages by cigarette smoke to release toxic oxygen radicals such as superoxide anion and hydrogen peroxide. Furthermore, alveolar macrophages from cigarette smokers are spontaneously cytotoxic for normal lung parenchymal cells. While the mechanism of how cigarette smoking leads to emphysema is likely very complex, these observations suggest that the proteases-antiprotease and oxidant-antioxidant balance in the lower respiratory tract play a major role in determining which individuals are susceptable to cigarette smoking induced disease.

Significance to Biomedical Research and the Program of the Institute: Destructive lung diseases are usually fatal and cause a significant amount of morbidity in our population. Studies of the lung destructive and therapeutic approaches show promise in terms of preventing these processes.

Proposed Course: Studies as outlined will be continued and the mechanisms of proteases and antiproteases in the lower respiratory tract evaluated in more detail. In addition, studies are being instituted to evaluate oxidant mechanisms as a cause of destruction in these disorders.

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ANNUAL REPORT OF THE

SURGERY BRANCH

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1983 through September 30, 1984

The Surgery Branch, NHLBI, since inception has had as a primary mission, the development of new or improved operations for treatment of patients with congenital and acquired heart disease. Concomitant with these historic achievements was the long-term observation of the results of surgical treatment by periodic assessment with traditional and new technologies. The laboratories of the Branch provided stimulus for new clinical investigations by thorough exploration of fundamental issues and innovations to ameliorate pathophysiology and test new pharmacologic agents and devices. This report encompasses these past goals and initiates new major clinical and laboratory programs.

Major accomplishments for the period included use of valve replacement and ultrasonic technology for idiopathic hypertrophic subaortic stenosis, initiation of study of coronary vascular tone after coronary artery bypass operations, a trial of mitral valve replacement without excision of the mitral valvular apparatus, continued evaluation of the efficacy and safety of investigational prosthetic valves in sheep, assessment and confirmation of benefit of the use of ultrasonic technologies in a variety of procedures, further development of a device to measure intramyocardial pH, evaluation of new adjunctive measures for myocardial preservation, and significant progress with the immune tolerance and cardiac transplantation program. Detailed long-term retrospective studies completed included: aortic valve replacement for aortic stenosis, evaluation of the treatment and pathology of tricuspid valvular disease and the experience with triple valve replacement. New studies have been initiated in laser technology, angioscopy, new synthetic valves, and new revascularization procedures.

Thirty-five projects completed, in progress, or recently initiated are reported upon. Extensive collaboration exists with traditional branches; i.e., Cardiology and Pathology, and new ones developed with the FDA, NASA, Hematology, Physics, Immunology, and scientists in the private sector. This summary of work is organized into two sections - clinical and laboratory programs and each subdivided by study title or generic headings. The guiding principles in these efforts are to critically evaluate clinical results and initiate new programs for improved surgical treatment based on thorough testing in the laboratory.

I. CLINICAL PROGRAMS

A. Idiopathic Hypertrophic Subaortic Stenosis (IHSS)

Patients with this genetically transmitted disease have long been of interest to both the Cardiology and Surgery Branches of NHLBI. An operation to relieve left ventricular obstruction was developed at NHLBI two decades ago and studies of palliative worth, new approaches, and use of new technology are illustrated below:

1. Mitral valve replacement in patients with IHSS

The standard operative treatment for relief of left ventricular outflow obstruction secondary to IHSS is a left ventriculomyotomy and myectomy (LVMM) and

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has been performed in more than 400 patients. Eleven patients have undergone mitral valve replacement as primary or secondary treatment of IHSS. Special considerations for mitral valve replacement in these patients include a thin septum-$\leq$ 18 mm (5 pts), reoperation for persistent obstruction following a LVMM (3 pts), mitral regurgitation secondary to endocarditis (2 pts) and atypical septal morphology (1 pt). No operative or late mortality has occurred. Six month evaluation of 4 patients has revealed good clinical and hemodynamic improvement. Mitral valve replacement will be considered in patients with obstructive IHSS under special circumstances but not as the preferred primary operative treatment.

2. Operative assessment of left ventriculomyotomy and myectomy using ultrasonic imaging

Intraoperative 2-D and M-mode echocardiographic displays and recordings have been used to determine septal thickness and morphology, point of mitral valve leaflet contact, and outlet geometry and orifice area in approximately 60 patients. This approach, especially important in patients with concomitant coronary disease in whom creation of a ventricular septal defect during myectomy is a significant hazard, has resulted in superior results. Intraoperative and postoperative hemodynamic studies show that remarkable palliation is achieved. All had postoperative pressure gradients of 15 mm Hg or less at rest. Significant gradients ($\geq$ 40 mm Hg) occurred with severe provocation only in that group that had severe obstruction at rest preoperatively. This trial will continue and use, in the future, new ultrasonic systems to quantitate pressure gradients and exit velocities noninvasively.

B. Prosthetic Valve Replacment: Long-term Results and New Approaches

1. Operative treatment of adults with aortic stenosis

A retrospective study was performed on 195 patients with isolated acquired or congenital aortic stenosis (AS) who had aortic valve replacement (AVR) during 1962-1971 ("decade I") and 1972-1981 ("decade II"). In both decades the majority of patients were male but older by 10 years and less symptomatic in the second decade. No significant difference resulted in operative results, long-term survival, or complications comparing patients with acquired versus congenital AS. A variety of Starr-Edwards, Bjork-Shiley, and Hancock prostheses were used and complications were related to specific valve types. Operative mortality was higher the first decade, 16% vs 3.4% for the second. Cumulative followup was 995 patient years for both decades. Good short and long-term symptomatic benefit resulted from AVR regardless of ventricular function or timing of operation. Long-term survival was similar at 5 years for both decades (65.8 $\pm$ 5% and 71.7 $\pm$ 6%) and was 59.1 $\pm$ 5% at 10 years for first decade patients. The first decade patients had a 73 $\pm$ 5% event free rate of thromboembolism at 5 years and 64 $\pm$ 6% at 10 years. Those operated upon during the second decade had a 76 $\pm$ 6% event free rate at 5 years. The incidence of freedom from an anticoagulation complication in the first decade was 89 $\pm$ 4% at 5 years and 80 $\pm$ 5% at 10 years; for those in the second, 87 $\pm$ 5% at 5 years.

2. The results of triple valve replacement at NIH

A retrospective analysis of 54 patients undergoing concomitant aortic, mitral, and tricuspid valve replacement at the NIH was performed. Pre and postoperative variables were subjected to statistical analysis. Patients were grouped by valve type to assess differences in long-term morbidity and mortality. Cumulative survival and event-free survival curves were compared. Findings included the

following: 1) operative survivors had a significantly lower preoperative mean pulmonary artery pressure; 2) decrease in pulmonary artery systolic pressure following operation had a positive correlation to long-term survival; 3) advanced age had a significant influence on operative mortality but patient sex and history of prior cardiac surgery did not; 4) patients with all porcine valves had an improved raw survival and event-free survival compared with other combinations of prosthetic valves.

3. Tricuspid valve disease and mitral regurgitation

To determine the incidence and severity of tricuspid disease in association with initial mitral valve disease we studied a group of 47 patients with pure, chronic mitral regurgitation with and without tricuspid valve regurgitation. Preoperative features and operative findings were statistically compared. The 22 patients without and the 25 patients with tricuspid regurgitation were similar except for the duration of symptoms. The latter group had a six year or more duration whereas those without tricuspid regurgitation came to surgical palliation much sooner. Palliation of symptoms was of longer duration in those without tricuspid regurgitation. These data suggest that postponement of mitral valve surgery after the onset of significant symptoms results in poorer prognosis and palliation.

4. Degenerative changes in tricuspid and mitral porcine bioprostheses

This study examined the relative rates of degeneration of bioprostheses in the tricuspid and mitral positions. Six patients had pairs of valves implanted and explanted simultaneously at a mean of 3.75 years. The major findings were degenerative changes in all 12 valves examined with an accelerated rate for bioprostheses in the mitral position.

5. Mitral valve replacement with and without chordal excision

The purpose of this clinical trial is to test the hypothesis that the entire mitral valve apparatus, if left intact at the time of valve replacement, will prevent the nearly uniform postreperfusion left ventricular dilation and low cardiac output associated with mitral valve replacement for mitral insufficiency of long duration. The results in 4 patients have been excellent in 3 and no change in the expected left ventricular failure in 1 patient. Two patients have had bioprostheses and 2 patients have had St. Jude prostheses. Matched paired patients having mitral valve replacement during the same interval have had subtle changes of left ventricular dysfunction in the postoperative interval which appear typical and different than the experimental group. Six month data will be obtained soon.

C. Congenital Heart Disease

1. Late results after operation for left ventricular outflow tract obstruction

More than 130 children and young adults with congenital forms of left ventricular outflow tract obstruction have been operated upon. The present study involves the late results of these operations. Patients included in the study were aged 1-18 years old who had been followed 5 or more years postoperatively. Forms of left ventricular outflow tract obstruction included valvular aortic stenosis, discrete and diffuse fibrous subaortic stenosis, and muscular subaortic stenosis. Operative mortalities were low (0-6%). However, the operations appear to be palliative only. Approximately 50% of patients have had satisfactory late results from the first operation.

2. Evaluation of right ventricular myocardium: morphologic and clinical relationships

Clinical studies in patients with congenital heart disease suggest that a "myocardial factor" related to dysfunction of heart muscle is responsible for unsatisfactory postoperative clinical courses in certain patients. No information is available concerning the time courses of morphological changes of myocardial hypertrophy in humans and the relationships to postoperative clinical results. Over the past 8 years, light and electron microscopic observations of right ventricular muscle biopsies have been compared to the clinical courses of patients undergoing operations for congenital heart anomalies associated with right ventricular systolic pressure overload. These observations have been summarized during the past year and have included additional patients under morphological and long-term clinical followup study.

D. Ischemic Heart Disease

1. Coronary vascular tone after coronary artery bypass operations

This clinical study tests the hypothesis that coronary blood flow dynamics of patients may be altered in the immediate interval after a coronary artery bypass procedure. Six patients have had complete studies. The results to date show wide variation of response to blood flow augmentation. In some, no significant changes in coronary vascular resistance, cardiac output, or any of the biochemical variables occur. Other patients show changes which can be related to less than optimal contractility. One patient demonstrated an increase in coronary vascular resistance prior to a decrease in cardiac output. This was reversed with a calcium channel blocking agent. The project continues.

II. LABORATORY PROGRAMS

A. Evaluation of Bioprosthetic Cardiac Valve Failure in Sheep

The purpose of this project is to develop an animal model of bioprosthetic cardiac valve failure to permit serial study of the pathologic alterations and hemodynamic dysfunction. Bioprosthetic valves implanted in juvenile sheep demonstrate accelerated but identical pathologic alterations of degeneration and calcification as those implanted in humans. More than 400 porcine aortic or bovine pericardial bioprosthetic valves from 6 different sources have been implanted in sheep. The characteristics of the pathologic changes in different types of clinical and investigational valves implanted in the mitral and tricuspid positions have been studied. New valves treated with processes to retard or eliminate the calcification process are under study. The model is additionally being utilized to validate ultrasonic techniques for the characterization of bioprosthetic valve flow profiles and for the noninvasive detection of valve failure.

B. Assessment and Use of New Ultrasonic Technologies

New modes of Doppler ultrasound and new signal conditioning of the received sonic spectrum permit accurate assessment of mean and peak velocities of blood flow. New systems will, in the near future, permit quantitation of the velocities of the entire flow field. Three studies using these technologies have been completed: 1) quantification of obstruction and regurgitation in right ventricular to pulmonary artery conduits with and without prosthetic valves; 2) quantification of aortic

insufficiency; 3) correlation of pressure drops across stenotic bioprosthetic valves in the mitral and tricuspid position. The results show high correlations by pressure drop by Doppler measurements to those measured simultaneously by traditional methods. Contrast echocardiography was successful in quantitation of aortic insufficiency. This noninvasive system has been used to determine the degree of regurgitation and obstruction in two patients to date.

C. Augmentation of Vascular Supply to Ischemic Myocardium

The purpose of this study is to determine the feasibility of augmenting myocardial blood flow by extracardiac methods. Specifically, the use of the internal mammary implant coupled to laser drilled ventricular channels and omental and/or splenic onlay wraps are considered. Additionally, the use of endothelial cell seeding together with growth factor may provide further augmentation. The rationale of the proposal is that many patients are not candidates for the coronary artery bypass procedure because of extreme atherosclerosis. Forty dogs, in various groups, are treated with combinations of the internal mammary implant, laser ventriculorophy, omental wrapping, and splenic translocations.

D. Myocardial Preservation Studies

A series of studies using the working isolated rat heart and open chest sheep have explored the significance of myocardial pH during ischemia and sought new pharmacologic measures to augment recovery. The NIH-developed fiberoptic system using membrane-captured phenolphthalin can measure pH in tissues continuously although the time constant is long at present. The effects of global normothermic myocardial ischemia on myocardial pH changes were studied in sheep. Recovery of function was enhanced when pH was preserved at 7.0 or greater. Continuous measurement of myocardial pH was a reliable predictor of recovery of left ventricular stroke work. In other studies, topical cardiac cooling and cardioplegic solutions were used. The preliminary data show a marked blunting of the time - pH decay with these modalities and accompanying improvement in recovery. The hearts of spontaneously hypertensive rats were used to evaluate the protective effect of pentobarbital during warm and cold global myocardial ischemia. After 35 minutes of warm (37°C) or 70 minutes of cold (20°C) global myocardial ischemia, pentobarbital-perfused hearts demonstrated better survival and recovery of function than controls. Amiodarone, a potent cardiotonic agent with unknown mechanisms of action, was similarly tested in the working isolated rat heart preparation at 37°C. The results show a narrow bandwidth dose response with maximal recovery of 70% of aortic flow at the 0.25 mg dose. In hypothermic studies, drug-treated hearts had significantly enhanced recovery of function vs. normothermic drug treated hearts.

E. Cardiac Transplantation: The Development of a Specific Immune Tolerance Model in Rhesus Monkeys

The purpose of this project is to test the hypothesis that immunologically mature rhesus monkeys transplanted with a genetically-mismatched heterotopic heart allograft at a time when the recipients' immune systems have been rendered incompetent with myeloblative total body irradiation will develop specific and permanent tolerance to the allografts. Myeloablative total body irradiation followed by autologous marrow transplantation after lymphocyte depletion of the marrow is the method employed to recreate a fetal immune environment. The development of an irradiated-monkey model with lymphocyte depletion of bone marrow has been accomplished.

F. Angioscopy, Laser Energy Characterization, and Use for Atherosclerotic Coronary and Peripheral Arteries

The use of ultrathin (1.5 - 1.8 mm) fiberoptic catheter systems for direct visualization of intraluminal anatomy of coronary arteries is under study. Ten sheep, placed on total cardiopulmonary bypass, have had angioscopes passed into the right or left coronary arteries. The coronary arteries are perfusion fixed using a silver chloride to stain endothelium. The amount of endothelial denudation injury is assessed by scanning and optic microscopy. The purpose of a second study is to define the most suitable laser source for vaporization of atherosclerotic plaque. Selected coronary arteries from human cadavers are exposed to lasers of various wave lengths and precise real time measurements of thermal diffusion are made by thermocouples and infra-red photography. Gross and histopathological techniques are used to compare the effects of various laser-tissue interactions. A colony of swine has been prepared with accelerated atherosclerotic lesions in the carotid and ilio-femoral arteries. Atherosclerotic plaques will be vaporized by laser energy delivered through a new fiberoptic delivery system.

G. In vivo Evaluation of a Synthetic Trileaflet Valve

During a 13 year program, a synthetic trileaflet valve has been developed. The leaflets are made from a micro-woven fabric which is highly flexible and elastic. Extensive durability and soft tissue implant studies were performed prior to mitral valve replacement in sheep. These data show that valvular insufficiency occurred early (24 hrs) which has been traced to alteration of filament geometry from stress compaction. New fabric designs have been completed and a second prototype is in production.

H. Blalock-Taussig Shunts with Prosthetic Grafts: Long-term Observation

The hypothesis tested in these studies was that prosthetic grafts of specific diameters, biomaterial composition, and lumenal topography have different long-term patency rates when used as subclavian to pulmonary artery conduits. Forty-six monkeys had catheterization at 3 months and 7 have been restudied at 1 year. Serial angiographic hemodynamic and ultrasonic measurements were made. Preliminary data show that dacron velour has a poor patency (40%) at 3 months. Bovine umbilical vein and bovine pericardium have a patency rate of 71% and 75% at 3 months. Expanded polytetrafluroethylene showed a patency rate of 82% at 3 months. The choice of conduit is highly important for long-term palliation of children with pulmonary oligemia.

I. Characterization of Subaortic Stenosis in Newfoundland Dogs

Genetically transmitted subaortic stenosis, absent at birth, has been studied in 176 animals, half of whom have hemodynamic and/or morphologic evidence for left ventricular outflow tract obstruction. The obstruction, similar to that in humans, is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow. This form of left ventricular outflow tract obstruction has proved to be a useful animal model to correlate Doppler predicted gradients with hemodynamically determined gradients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02695-02 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Evaluation of the RV myocardium: Morphological and clinical relationships

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

Victor J. Ferrans, M.D., Ph.D., Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Pathology Branch, NHLBI

LAB/BRANCH

Surgery Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

xxx ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Clinical studies in patients with valvular heart disease and with congenital heart disease suggest that a "myocardial factor" related to dysfunction of the heart muscle itself is responsible for unsatisfactory postoperative clinical courses in certain patients. Additionally, controversies exist whether to perform palliative operations or anatomically corrective operations upon patients with congenital heart anomalies. The proper timing of operations is questioned as well. No information is available concerning the time courses of morphological changes of myocardial hypertrophy in humans and the relationships of morphological changes to postoperative clinical results.

Over the previous eight years we have compared light and electron microscopic observations with clinical courses of patients undergoing operations for congenital heart anomalies associated with right ventricular systolic pressure overload. We have published descriptions of myocardial morphological alterations of right ventricular hypertrophy and degeneration in these patients during previous years. During the past year we have summarized those observations and have included additional patients under morphological and long-term clinical follow-up study.

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Project Description: Based upon our studies, we generalize that patients with congenital heart disease and right ventricular hypertrophy, who are over 15 to 20 years old, may experience a less than optimal postoperative clinical course.

At present we have studied by light and electron microscopy the myocardium obtained at operation from 75 patients with right ventricular pressure overload (tetralogy of Fallot physiology). The patients have been divided into four groups: I (36 patients) aged 10 months to 10 years; II (22 patients) aged 11 to 20 years; III (8 patients) aged 21 to 29 years; and IV (9 patients) aged 30 to 53 years.

Half of group III and 78% of group IV patients suffered clinically apparent cardiac failure, major arrhythmias, peri-operative mortality, and/or late deaths. Alterations of cardiac hypertrophy and degeneration were severe in both groups of patients. Prominent interstitial fibrosis was observed in groups I, II, III, and IV at frequencies of 19%, 23%, 25% and 100% respectively. Myofibrillar lysis, myelin figures, smooth endoplasmic reticulum proliferation, and cell membrane associated spherical macroparticles were less common in groups I, II, and III (0% to 25%) than in group IV (67% to 100%). Cellular atrophy, disorganization of cells and myofibrils, lipid accumulation, intracytoplasmic junctions, and thickened basal laminae were frequent in cardiac muscle from groups III and IV.

Thus, the degenerative morphological alterations of chronic right ventricular hypertrophy in man appeared related to clinical myocardial dysfunction. These relationships have implications for patients' long-term prognoses, the type and timing of their operations, and their requirements for intraoperative myocardial protection.

Proposed Course: (1) Tissues obtained from six additional patients in groups III and IV (over age 20 years) will be evaluated and compared with the patients' clinical courses. (2) We have right ventricular myocardial biopsies from 37 additional patients in groups I and II (age 3 days to 20 years). We will subdivide these patients by ages: 3 days to 5 years, 6 to 10 years, 11 to 15 years, and 16 to 20 years for study. Following this, the total number of patients in all groups, which we have studied will be 118. We anticipate that the information obtained from the study of all patients with right ventricular systolic overload will allow recommendations regarding the ideal time for palliative or anatomically corrective operations. (3) Approximately 70 patients are available for a five year follow-up study, which is planned in the next year. (4) A non-invasive evaluation of right ventricular function by two dimensional real time echocardiography and by radionuclide angiography is planned in cooperation with the Cardiology Branch. This evaluation of right ventricular function will be compared to our morphological evaluation of the patients' right ventricular myocardium.

Publications:

Jones M, Ferrans VJ: Myocardial ultrastructure in children and adults with congenital heart disease. In: Congenital Heart Disease in Adults, 2nd Edition (Roberts WC ed). F.A. Davis, Philadelphia. In Press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01HL 02697-05 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of subaortic stenosis in Newfoundland dogs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

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Lilliam M. Valdes-Cruz, M.D., Guest Worker, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

Pathology Branch, NHLBI
Section of Lab Animal Medicine & Surgery, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been characterizing the morphologic and the hemodynamic abnormalities occurring in Newfoundland dogs with genetically transmitted subaortic stenosis. Of 176 animals studied this far, almost one-half have hemodynamic and/or morphologic evidence for left ventricular outflow tract obstruction. The obstruction is absent at birth, appearing after one month of age. The lesion presents clinically by the presence of a precordial murmur, thrill, arrhythmia, congestive heart failure, bacterial endocarditis of the aortic valve, or sudden death. Obstruction to left ventricular outflow is caused by the development of a circumferential, subaortic fibromuscular ring continuous with the anterior leaflet of the mitral valve, virtually identical to the same lesion in humans. The obstruction is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow. This form of infra coronary left ventricular outflow tract obstruction has proved to be a useful animal model to correlate Doppler predicted gradients with hemodynamically determined gradients.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description: In the 176 dogs studied hemodynamic and/or morphologic evidence of LV outflow tract obstruction (LVOTO) was present in 66 animals, including 25% of 77 1 year old, 38% of 76 aged 13-24 months, 73% of 30 aged over 24 months. None of 22 newborns and 1 of 5 1 month old had LVOTO. No dogs had assymmetrical septal hypertrophy or myocardial cellular disarray. LVOTO was associated with LV hypertrophy (LVbody weights 4.5 g/kg), sudden death (n=16), thickened aortic valves, bacterial endocarditis (n=9), intramural coronary artery lesions, myocardial fibrosis and abnormalities of myocardial blood flow. All dogs with LVOTO had circumferential subvalvular fibrous rings continuous with the ventricular septum and anterior mitral leaflet. Of 64 dogs without LVOTO the septal endocardium was thickened in 38%. Although present in a few dogs without LVOTO, an unusual angulation of the aorta and the septum occurred in dogs with LVOTO.

Continuous mode Doppler interrogation with two dimensional echo guidance was used in 23 dogs to compare the angle corrected Doppler maximal velocities to pressure gradients across the obstruction at rest and after pharmacological provocation. Doppler-predicted and measured pressure gradients were closely correlated ($r = 0.95$, SEE = 7.1 mm Hg), thus validating the predictive capability of Doppler for estimating pressure gradients in this animal model resembling infra coronary left ventricular outflow tract obstruction in humans.

Subaortic stenosis and its sequelae in Newfoundland dogs appears to be acquired after birth and its pathogenesis to be due to the interaction of genetic, morphologic, and hemodynamic factors.

Proposed Course: Investigations are on-going to further characterize this lesion and to compare/correlate it with infra coronary left ventricular outflow tract obstruction in humans. Doppler tracking studies are being performed to follow the development and progression of the outflow tract obstruction.

Presentations:

Valdes-Cruz: Prediction of the gradient in fibromuscular subaortic stenosis by continuous wave 2D Doppler echocardiography: Animal studies. 56 Annual Scientific Session, American Heart Association, November 1983.

Publications:

Valdes-Cruz LM, Pierce JE, Sahn DJ, Scagnelli S, Hunter P, Jones M: Prediction of the gradient in fibromuscular subaortic stenosis by continuous wave Doppler 2D echocardiography. Circulation 68 (Supp III): III-36, 1983.

Valdes-Cruz LM, Jones M, Scagnelli S, Sahn DJ, Tomizuka FM, Pierce JE: Prediction of gradients in fibrous subaortic stenosis by two-dimensional echo and continuous wave Doppler: Animal studies. submitted for publication

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02714-04 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Evaluation of bioprosthetic cardiac valve failure in an animal model

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

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Lilliam M. Valdes-Cruz, Guest Worker, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

Pathology Branch, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
10	5	5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

The purpose of this project is to develop an animal model of bioprosthetic cardiac valve failure and to utilize this animal model system to evaluate the pathologic alterations and hemodynamic dysfunction which develop in the valves. We have shown that bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans; however, the development of these alterations is accelerated in sheep as compared to humans. Nearly 300 porcine aortic or bovine pericardial bioprosthetic valves from six different sources have been implanted in the animal model system to assess the characteristics of the pathologic changes, to compare the alterations in different types of valves, to compare the alterations occurring in valves implanted in the mitral versus the tricuspid positions, and to evaluate valves treated prior to implantation with processes to retard or eliminate the calcification process. The model is additionally being utilized to validate ultrasonic techniques (Doppler/2-D echocardiography) for the characterization of bioprosthetic valve flow profiles and for the detection non-invasively of valve failure.

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Project Description: Although more than 20 years have passed since the first prosthetic cardiac valves were implanted in humans, the development of an ideal cardiac vavle substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions and for certain subgroups of patients, primarily because they do not require chronic anticoagulant therapy. However, it is now apparent that the long-term durability of bioprosthetic cardiac valves is finite; degeneration and calcification of these valves are the major complication of long-term implantation.

An *in vivo*, *in situ* investigational model is essential for the study of the mechanisms of degeneration and calcification in substitute bioprosthetic cardiac valves. For this purpose we have the following criteria in an animal model system developed in domestic sheep (*Ovis aries*): (1) the bioprostheses should be implanted in growing animals to simulate the physiologic conditions in young humans, in whom accelerated degenerative alterations of bioprosthetic valves are known to occur; (2) the pathologic alterations should develop within several months after implantation to permit expeditious study; (3) at maturity the animals should not outgrow the bioprosthetic cardiac valves; (4) the animals must be of suitable sizes for standard cardiopulmonary bypass techniques; and (5) the pathologic alterations in the bioprosthetic valves must be similar in the animals and in humans.

During the past four years an animal model system has been developed in juvenile (10-15 week old) sheep which permits early and late studies of host-prosthesis interactions. Over 300 porcine aortic valves (PAV) and bovine pericardial valves (BPV) have been implanted in the tricuspid (TVR) or mitral (MVR) positions. Long-term survival (30 days - 1 year) in the last 200 animals has been 85%. In analyzing the results obtained with valve implantations in this model system, emphasis has been given to: (1) early and late hemodynamic investigations of valve function; (2) pathologic alterations, including localization of calcific deposits; (3) quantitative analyses of valve calcification; (4) evaluations of treatment processes to decrease valve calcification; and (5) use of intracardiac Doppler/2-D echocardiography to study valve function.

Early hemodynamic studies showed anticipated performance for the manufacturer's size 25 mm valves utilized:

	Mean Gradient (mm Hg)	Gorlin Valve Area (mm Hg)
TVR - BPV	4.9 ± 0.3 (n = 61)	2.0 ± 0.2 (n = 61)
TVR - PAV	5.4 ± 0.2 (n = 84)	1.6 ± 0.1 (n = 84)
MVR - BPV	6.8 ± 0.7 (n = 46)	1.0 ± 0.2 (n = 46)
MVR - PAV	8.9 ± 0.3 (n = 129)	1.8 1 0.1 (n = 126)

Cardiac outputs = 2.85 ± 0.04 L/min (n = 360). At the time of explantation (average 5 months) hemodynamic observations reflected the degree of pathologic alterations. Pathologic changes resembled those found in humans, including perforations; microthrombi; red blood cell, round cell and giant cell infiltrations; fibrous sheathing with and without cuspal retraction and commissural fusion; insudation of plasma proteins; cuspal delamination; infection; presence of cotton fragments; strut creep; cuspal abrasion and perforation by sutures;

and calcific deposits. Morphologic sites of calcific deposits included cuspal connective tissue and cuspal cells, the muscle shelf and aortic wall of PAV's, as well as microthrombi, vegetations and fibrous sheaths. Cartilage and bone formation have also been observed. Quantitative analyses of calcium content demonstrated that standard valves contained 121.0 ± 12.6 mg/g cuspal dry weight (n = 291 cusps); whereas, unimplanted valves contained 1.0 ± 0.1 mg/g (n = 70 cusps). There was a trend, not statistically significant, for calcium to be greater in MVR's than TVR's and in BPV's than in PAV's. Studies of valves treated prior to implantation to decrease calcific deposits have shown mitigation of valvular calcification. The model is being evaluated utilizing pulsed/continuous Doppler interrogation with 2-D echocardiographic guidance (IREX scanner) for validation of noninvasive determinations of gradients, areas and regurgitation of bioprosthetic valves.

Thus, implanting bioprosthetic valves in young sheep provides an excellent in vivo, in situ means for investigating the bioprosthetic valves' preparation and design.

Proposed Course: On-going studies include a comparison of the various types of valves, a comparison of the alterations occurring in the mitral versus the tricuspid positions, evaluations of methods to ameliorate or eliminate the degenerative processes, validation of ultrasonic techniques for evaluating pathophysiologic abnormalities produced by the valves, and development of a synthetic leaflet valve.

Presentations:

Jones M: Mitigation of calcification of Carpentier-Edwards bioprosthetic cardiac valves. 33rd Annual Scientific Session, American College of Cardiology, Dallas, TX, March 1984.

Valdes-Cruz LM: Continuous wave Doppler estimates the severity of stenosis of bioprosthetic valves in the mitral and tricuspid positions: Studies in a chronic lamb model. 33rd Annual Scientific Session, American College of Cardiology, Dallas TX, March 1984.

Jones M: In vivo experimental evaluation of bioprosthetic cardiac valves. AAMI 19th Annual Meeting.

Jones M: Morphology of calcific deposits in implanted cardiac valvular bioprostheses. AAMI 19th Annual Meeting.

Publications:

Arbustini E, Jones M, Ferrans VJ: Formation of cartilage in bioprosthetic cardiac valves implanted in sheep. A morphologic study. Am J Cardiol 52:632-636,1983.

Arbustini E, Jones M, Moses RD, Eidbo EE, Carroll RJ, Ferrans VJ: Modification by the Hancock T6 process of calcification of bioprosthetic cardiac valves implanted in sheep. Am J Cardiol 53:1388-1396, 1984.

Valdes-Cruz LM, Jones M, Main JC, Eidbo EE, Sahn DJ, Swensson RE, Kashani IA, Elias W: Continuous wave Doppler estimates the severity of stenosis of bioprosthetic valves in the mitral and tricuspid positions. J Am College Cardiol 3:493, 1984 (abstr)

Jones M, Arbustini E, Applebaum RE, Eidbo EE, Ferrans VJ: Mitigation of calcification of Carpentier-Edwards bioprosthetic cardiac valves. J Am College Cardiol 3:486, 1984 (abstr)

Jones M, Arbustini E, Eidbo EE, Ferrans VJ: In vivo experimental evaluation of bioprosthetic cardiac valves. Proceedings AAMI 19th Annual Meeting: 40, 1984. (abstr)

Ferrans VJ, Arbustini E, Eidbo EE, Jones M: Morphology of calcific deposits in implanted cardiac valvular bioprostheses. Proceedings AAMI 19th Annual Meeting: 26, 1984. (abstr).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02716-04 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Late results after operations for left ventricular outflow tract obstruction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

Moses, Robert D., M.D., Medical Staff Fellow, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.25	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have operated upon more than 130 children and young adults for congenital forms of left ventricular outflow tract obstruction. The present study involves the late results of these operations performed at our institution and a review of the published results from other institutions. Patients included in the study were aged 1-18 years old at operation who had been followed five or more years postoperatively. Forms of left ventricular outflow tract obstruction included valvular aortic stenosis, discrete and diffuse fibrous subaortic stenosis, and muscular subaortic stenosis. Operative mortalities were low (0-6%). However, the operations appear to be palliative ones because only approximately 50% of patients have satisfactory late results.

938

Project Description: We evaluated the late results of operations for the relief of left ventricular outflow tract obstruction (LVOTO) in young patients, 1-18 years old, from our institution who have been followed for at least five years and from studies in the recent literature which had average follow-up durations of five or more years. Operative mortalities for our series and those series we reviewed were low: 1.9% of 522 patients with valvular aortic stenosis (VAS), 6.0% of 222 patients with fixed subvalvular aortic stenosis (SAS) and 5.5% of 18 patients with hypertrophic subaortic stenosis (HSS). From our series, gradients early postoperatively were decreased to less than 40 mm Hg in 88% (30/34) with VAS, in 68% (15/22) with SAS and in 88% (8/9) with HSS. Late survivals for patients in the combined series were: 90% (472/522) for VAS, 86% (190/222) for SAS and 82% (14/17) for HSS, after mean follow-up periods of 5-14.4 years. All of our late survivors have had symptomatic improvement; 95% (58/61) are asymptomatic. However, for our patients actuarial analysis predicts that 40 ± 8% of those with VAS and 44 ± 10% of those with SAS after ten years will be free from the adverse postoperative events of residual and/or recurrent LVOTO, clinically significant aortic regurgitation, reoperation, endocarditis, or late death. Using the same adverse postoperative events to determine satisfactory late results from the combined series, we found that 54% (381/522) of those operated upon for VAS, 54% (120/222) of those operated upon for SAS and 78% (14/18) of those operated upon for HSS had satisfactory late results 5-14 years after operation. Of our patients having unsatisfactory late results, major hemodynamic abnormalities were detected in 55% (23/42) within one year postoperatively. Thus, it appears that operations for most children with LVOTO are palliative ones. These patients should have early postoperative assessments and continuing long-term follow-up evaluations during childhood, adolescence, and adulthood.

Proposed Course: The patients in this study will continue to be followed and additional patients will be added to the study as they undergo operations. The purpose of the on-going studies is to further delineate the natural history of these operatively treated cardiac abnormalities with the objectives of eliminating or alleviating unsatisfactory late results.

Publications:

Jones M, Barnhart GR, Morrow AG: Late results after operation to relieve obstruction to left ventricular outflow. In: Congenital Heart Disease after Surgery. Benefits, Residua and Sequelae. (Engle MA, Perloff JK, Eds):105-132, Yorke Medical Books, New York, 1983.

Jones M.: Results after valvulotomy for congenital aortic stenosis in children and adolescents. In: Aortic Valve Stenosis and Aortic Regurgitation. (Roberts WC, Ed.) Yorke Medical Books, New York. In press.

Moses RD, Barnhart GR, Jones M: The late prognosis after localized resection for fixed (discrete and tunnel) left ventricular outflow tract obstruction. J. Thorac Cardiovasc Surg 87:410-420, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02725-02 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The protective effect of pentobarbital during warm and cold myocardial ischemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Greg H. Ribakove, M.D., Guest Worker, Surgery Branch, NHLBI

Mark P. Schlesinger, M.D., Guest Worker, Surgery Branch, NHLBI
Michael J. Rubeis, B.S., Laboratory Technician, Surgery Branch, NHLBI
James P. Voigtlander, B.S., Laboratory Technician, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The protective effect of pentobarbital during warm and cold global myocardial ischemia was evaluated in an isolated working heart model using the hypertrophied hearts of spontaneously hypertensive rats. After 35 minutes of warm (37°C) or 70 minutes of cold (20°C) global myocardial ischemia, pentobarbital perfused hearts demonstrated better survival than controls. Thus, pentobarbital ameliorates the deleterious effects of warm and cold ischemia in hypertrophied hearts.

940

Project Description: The protective effect of pentobarbital during warm (37°C) and cold (20°C) global myocardial ischemia was evaluated in an isolated working heart model using 350 - 450 gm Spontaneously Hypertensive (SHR) rats. Hearts of these rats were hypertrophied (dry heart/body weight = 1.2 + 0.03 mg/g, n = 38) as compared to normal Sprague-Dawley rats of the same weight and age (dry heart/body weight = 0.70 ± 0.02 mg/g, n = 22) ($p < 0.01$). Heart rate, aortic pressure, coronary sinus flow and aortic output were measured before and after a 35 minute warm or a 70 minute cold ischemic period. The experimental hearts were perfused with 12 ml of a 1.2 mM solution of pentobarbital in 0.9% saline at 80 mm Hg immediately prior to the onset of ischemia. The control hearts were perfused with 0.9% saline alone. In the warm ischemic group 10 of 10 experimentals (100%) versus 4 of 10 controls (40%) re-established any cardiac output (survived) after ischemia ($p < 0.02$). The average preischemic aortic output for warm controls was 53 ± 3 ml/min and for warm experimentals was 52 ± 4 ml/min. Postischemic aortic output dropped to 8 ± 3 ml/min for warm controls and 32 ± 4 ml/min for warm experimentals, representing a 15% and 62% recovery of function respectively ($p < 0.01$). In the cold ischemic group, 5 of 6 experimentals (83%) versus 0 of 6 controls (0%) survived ischemia ($p < 0.01$). The average preischemic aortic output for cold controls was 59 ± 4 ml/min and for cold experimentals was 52 ± 5 ml/min. Postischemic aortic output dropped to 31 ± 7 ml/min for cold experimentals representing a 60% recovery of function, while none of the cold controls recovered any cardiac output. All data are expressed as a mean ± the standard error. These results demonstrate that pentobarbital ameliorates the deleterious effects of warm and cold ischemia in hypertrophied hearts.

Proposed Course: The efficacy of pentobarbital as a cardioplegic agent will be evaluated in sheep on cardiopulmonary bypass without, and possibly with, hypothermia. This will include both acute studies, in which intraoperative myocardial biopsies will be taken for high-energy phosphate analysis, and chronic studies, involving comparison of pre and postoperative hemodynamic performance.

Presentations:

Ribakove, GH: The protective effect of pentobarbital during warm and cold ischemia. American College of Surgeons, November 1983.

Publications:

Ribakove GH, Schlesinger MP, Rubeis MJ, Jones M: The protective effect of pentobarbital during warm and cold ischemia in hypertrophied hearts. Surg Forum XXXIV:327-329, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02727-02 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Experimental Pericarditis: Ultrastructural Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Steven R. Cohen, M.D., Clinical Associate, Surgery Branch, NHLBI

Lee Leak, Ph.D., Professor of Anatomy, Howard University
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Victor Ferrans, M.D., Ph.D., Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

Pathology Branch, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	3/4	1/4

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We injected the pericardial cavities of 25 sheep with either a killed bacterial or suspension or a control solution to produce pericarditis. The sheep have been studied at intervals of 3 hours to 1 year after injection. In vitro glutaraldehyde fixation of the epicardium and pericardium was performed. Epicardial and pericardial tissues were studied by light and electron microscopy. Killed bacteria produced acute and chronic inflammatory changes within the pericardial cavities. The epicardium demonstrated greater inflammation and necrosis than the pericardium, suggesting that a major component of acute and chronic "pericarditis" is related to alterations in the epicardium.

942

Project Description: Thirty sheep have been injected with either (1) killed staphylococcal aureus with saline and Freund's adjuvant; (2) Freund's adjuvant and saline + saline; (3) saline alone; (4) no injection. Those receiving killed bacteria with adjuvant and saline were sacrificed at 3, 6, 8, 24, 36, 48 hours and 6 days, 8 days, 2 weeks, one month, 9 months, one year and 1-1/2 years. Those receiving control injections were sacrificed at 6 hours, 24 hours, one month and 9 months. All animals underwent acute thoracotomy at the time of sacrifice. Without arresting the heart or incising the pericardium, the aorta and the superior vena cava were cannulated. Subsequently, the aorta was cross-clamped and heparin was injected into the superior vena cava. The heart was arrested with potassium chloride. The heart was immediately fixed with glutaraldehyde perfused via the coronary arteries. Then the pericardium was perfused with glutaraldehyde via a cannula inserted into the pericardial cavity. The entire heart and intact pericardium was excised and immersed in glutaraldehyde. Specimens of the myocardium and pericardium were prepared for light and electron microspic evaluation. The reaction appeared most severe in the epicardium with necrosis and inflammation.

Proposed Course: Upon completion of morphologic analysis, we plan publication of our observations. A study of the lymphatic clearance of inflammatory cells in pericarditis is planned. Manuscript is in preparation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02731-02 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Operative assessment and results of left ventriculomyotomy and myectomy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI

Barry Maron, M.D., Senior Investigator, Head, Echo Lab, Cardiology Branch NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

XXX ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A standard myotomy and myectomy has been performed for relief of left ventricular outflow tract obstruction secondary to asymetric septal hypertrophy (ASH) in 410 patients. Fifty-eight patients have been operated upon and an attempt has been made to tailor the operative approach depending upon septal thickness, distribution, level of systolic anterior motion contact of septum and concomitant coronary artery disease. Intraoperative 2D and M-mode echos have been performed on a number of these patients providing precise data utilized intraoperatively. Patients with concomitant CAD represent a higher risk for VSD creation which may be avoided by a modified left ventricular myotomy and myectomy or mitral valve replacement. Hemodynamic data are presented based on preoperative resting gradients 0-50 mm Hg (n = 13) and > 50 mm Hg (n = 45). Postoperative hemodynamic studies reveal good relief of resting gradient in most patients but significant provocable gradients remain in some patients, especially in those with preoperative resting gradients > 50 mm Hg. Medical therapy is continued in patients with significant provocable gradients following operative palliation.

944

Project Description: Four-hundred ten patients have undergone operative treatment for resting or provocable left ventricular outflow tract gradients secondary to ASH. The operation performed has been the classic Morrow operation(left ventriculomyotomy and myectomy) in 399 patients and mitral valve replacement in 11 patients. Choice of operative approach is based upon echo examination of the septum regarding septal thickness, distribution of septal hypertrophy, i.e. localized or homogeneous and contact point on septum of anterior leaflet. The preoperative M-mode and 2-dimensional echo may vary depending upon body habitus providing less than precise morphology. Patients now routinely have an intraoperative echo with the probe placed directly on the heart for more exact measurements and the operative approach is planned based on these data. A post resection echo is also performed which has been helpful in predicting relief of outflow tract obstruction. Septal variation in thickness and distribution of hypertrophy especially in patients with concomitant CAD may warrant a modified operation to avoid complications or to provide maximal relief of left ventricular outflow tract obstruction.

Patients were placed in two groups based on preoperative hemodynamic data. Group I includes patients with $\geq$ 50 mm Hg resting gradients and Group II includes patients with 0-50 mm Hg resting gradients, who are generally recommended for operation because of significant provocable gradients. The last 27 patients have had myocardial protection using cold cardioplegia rather than moderate hypothermia (26-30°C) flaccid arrest. The left ventricular myotomy-myectomy has been altered only in depth and thickness of $\leq$ 18 mm. A thin septum ($\leq$ 18 mm) currently warrants a mitral valve replacement for maximal relief of resting or provocable LVOT obstruction.

Group I - Patients with > 50 mm Hg Resting Gradients ± CAD

Forty-five patients (22 males; 25 females) having resting gradients $\geq$ 50 mm Hg have undergone operation for relief of LVOT obstruction secondary to ASH. The average age at time of operation for males was 56.0 ± 16 (range 22-81) and for females 52.2 ± 17 (range 19-70). There were 4 early deaths (< 30 days;8.9%); 2 late (>30 days) deaths (4.8%). Early deaths were attributed to low cardiac output (n=3) and mediastinitis (n=1); late deaths to arrhythmia (n=1) and sepsis (n=1). Left ventricular myotomy and myectomy was performed in 39 patients, mitral valve replacement in 6 (2 as primary operative treatment; 4 in patients with persistent obstruction following Morrow procedure). Other operations performed included aortic valve replacement (n=2); AVR + CABG (n=1); tricuspid valve replacement (n=1) and closure of iatrogenic VSD (n=1).

Pre and Postoperative Hemodynamic Data

	Pre (n)	Post (n)
PAW	15.4 ± SE 7 (n=43)	13.7 ± 5 (n=22)
LVEDP	17.6 ± 8 (39)	15.8 ± 6 (30)
Gradients		
Rest	90.0 ± 4.7 (44)	13.1 ± 4.1 (36) 0 gradient n=19

945

* Valsalva	102.2 ± 7 (18)	45.1 ± 6 (34) 0 gradient n=3
Amyl nitrite	97.5 ± 6 (10)	62.0 ± 6 (32) 0 gradient n=0
Isuprel	115 ± 6 (5)	76.8 ± 8 (29) 0 gradient n=0

* Patients with rest gradients > 100 mm Hg not provoked

Group II - Patients with 0 to ≤ 50 mm Hg Resting Gradients ± CAD

Thirteen patients (9 males; 4 females) were found to have resting gradients of 0 to ≤ 50 mm Hg. The average age at operation was 55.3 ± 14 (range 22-64) for males and 53.5 ± 8 for females (range 46-85). There was one early death (7.7%) secondary to persistent bleeding and hypotension in a patient who had undergone a previous CABG and one late death (10%) attributed to congestive heart failure in a patient with a dilated cardiomyopathy with only a provocable gradient. There were two iatrogenic VSD, one closed at initial operation (pt died) and one occurring late closed at 7 days (pt alive), and one patient developed complete heart block requiring a pacemaker. Twelve patients had the Morrow procedure performed and one a MVR.

Pre and Postoperative Hemodynamic Data

	Pre (n)	Post (n)
PAW	13.8 ± 8 (11)	11.1 ± 4 (7)
LVEDP	16 ± 9 (11)	12.3 ± 6 (8)
Gradients		
(1) Rest = 0 (n=8)		
Valsalva	56.3 ± 29 (8)	35.0 ± 22 (3)
Amyl nitrite	66.9 ± 23 (8)	33.8 ± 27 (4)
Isuprel	108.1 ± 30 (8)	53.8 ± 51 (4)
(2) Rest gradient > 0 mm Hg but ≤ 50 mm Hg (n=5)		
Valsalva	95 ± 23 (5)	15 ± 7 (2)
Amyl nitrite	103.8 ± 19 (4)	36.6 ± 25 (3)
Isuprel	98.3 ± 38 (3)	31.7 ± 25 (3)

Patients with resting gradients > 50 mm Hg may expect excellent relief of outflow tract obstruction following the Morrow procedure but may have residual provocable gradients. Medical therapy is recommended in patients with significant (> 50 mm Hg) provocable gradients. Symptomatic improvement may not reflect hemodynamic data in that a majority of patients are functional class I-II at six months. Patients undergoing operation primarily for provocable gradients will have smaller postoperative gradients but a small percentage will provoke to 50 mm Hg. Patients with persistent resting or provocable obstruction who remain symptomatic and require further operative intervention

should be considered for MVR.

Proposed Course: Intraoperative investigations will be continued to further define septal morphology to tailor the operation to the individual patients for maximal relief of LVOT obstruction performed at the lowest operative risk.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02733-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mitral valve replacement in patients with IHSS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI

Barry Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The standard operative treatment for relief of left ventricular outflow obstruction secondary to idiopathic hypertrophic subaortic stenosis is a left ventriculomyotomy and myectomy and has been performed in 400 patients. Eleven patients have undergone mitral valve replacement as primary or secondary treatment of IHSS. Special considerations for mitral valve replacement in these patients include thin septum - ≤ 18 mm (5 pts), reoperation for persistent obstruction following a left ventriculomyotomy and myectomy (3 pts), mitral regurgitation secondary to endocarditis (2 pts) and atypical septal morphology (1 pt). A low profile prosthesis was used in these patients, 8 Bjork-Shiley and 3 Hancock porcine bioprostheses. No operative or late mortality has occurred and 1 patient required a closure of a perivalvular leak 6 months after MVR. Six month evaluation of 4 patients has revealed good clinical and hemodynamic improvement. Mitral valve replacement should be considered in patients with obstructive IHSS under special circumstances but not as the preferred operative treatment.

948

Project Description: The standard operative treatment for relief of left ventricular outflow obstruction secondary to idiopathic hypertrophic obstruction is a left ventriculomyotomy and myectomy which has been performed on approximately 400 patients in the Surgery Branch. The spectrum of this obstructive cardiomyopathy is being more precisely defined by the use of intraoperative M-mode and 2D echos. These echo data are important in planning an operative approach with the lowest mortality and morbidity while achieving maximum relief of resting and provocable left ventricular outflow tract obstruction.

Special considerations which have evolved over a 2 year period which warranted a mitral valve replacement rather than a left ventriculomyotomy and myectomy include: (1) a septum $\leq$ 18 mm in thickness in the region of resection; (2) atypical septal morphology; (3) mitral regurgitation secondary to endocarditis; and (4) a previous resection with persistent symptoms due to incomplete relief of obstruction. A septum of $\leq$ 18 mm precludes removing the usual amount of muscle and increases the risk of producing an iatrogenic ventricular septal defect, especially in the presence of coronary artery disease. Atypical septal morphology, such as an unusual low lying obstruction (> 4.0 cm below aortic anulus) makes the distal resection difficult since it cannot be performed under direct vision. Patients with severe MR secondary to endocarditis are compromised by both obstruction and regurgitation which are relieved by MVR. Patients with residual obstruction often have a relatively thin septum in the region of previous LV myotomy-myectomy placing the patient at a greater risk for an iatrogenic VSD.

Eleven IHSS patients (5 males, 6 females) whose mean age was 49.9 years (range 25-71) have undergone MVR, 5 for thin septums, 1 for atypical septal morphology, 2 for mitral regurgitation with ruptured chordae tendinea secondary to endocarditis and 3 for persistent symptoms and demonstrated resting obstruction. All patients were functional class III preoperatively. There have been no operative or late deaths. One patient required closure of a perivalvular leak 6 months after MVR. Bjork-Shiley prostheses were used in 8 patients and Hancock porcine bioprostheses in 3. Patients having a Bjork-Shiley prosthesis receive indefinite anticoagulation with sodium warfarin. Those with a Hancock valve do not.

Three patients having thin septums have returned for postoperative evaluation and all are functional class I. Preoperative resting gradients averaged 73 mm Hg, 118 mm Hg with Valsalva, 100 mm Hg with amyl nitrite and isuprel. Postoperatively there was no resting gradient in these three patients and the highest provocable gradient was 25 mm Hg. Cardiac output/index was 4.8/2.8 preoperatively and 4.9/2.5 postoperatively in these patients. One patient undergoing MVR following a previous LV M&M had a resting gradient of 70 mm Hg which was 5 mm Hg postoperatively and provoked to 15 mm Hg with a cardiac output/index of 5.2/3.6 preoperatively and 4.0/2.6 postoperatively. The remaining 7 patients have not returned for postoperative evaluation at this time.

Mitral valve replacement for relief of resting and provocable left ventricular outflow tract obstruction is effective and avoids the complication of an iatrogenic VSD in a patient with a thin septum. Previously operated patients with persistent obstruction following LVMM will be benefited by MVR; although

small provocable gradients may persist. Patients having IHSS and moderate to severe MR secondary to endocarditis should undergo MVR.

Proposed Course: Investigations are ongoing to further define a subgroup of patients who will have a MVR rather than a LVMM as primary treatment of their obstructive cardiomyopathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02734-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Operative treatment of adults with aortic valve stenosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A retrospective study was carried out of 195 patients having hemodynamic significant isolated acquired or congenital aortic stenosis (AS) undergoing aortic valve replacement (AVR) during 1962-1971 ("decade I') and 1972-1981 ("decade II"). Coronary artery disease did not exclude a patient from the study unless a left main lesion was present and simultaneous revascularization was not performed. In both decades the majority of patients were male but older by 10 years and less symtpomatic in the second decade. No significant difference resulted in operative results, long-term survival, or complications comparing patients with acquired versus congenital AS. A variety of Starr-Edwards, Bjork-Shiley, and Hancock prostheses were used and complications were related to specific valve types. Operative mortality was higher the first decade, 16% vs 3.4% the second. Cumulative followup was 995 years for both decades with good short and long-term symptomatic benefit resulting from AVR regardless of ventricular function or timing of operation. Long-term survival was similar at 5 years for both decades (65.8 ± 5% and 71.7 ± 6%) and was 59.1 ± 5% at 10 years for first decade patients. Thromboembolism and anticoagulation complications were the most frequent clinical events following AVR. Of patients operated upon the first decade 73 ± 5% were event free of thromboembolsim at 5 years and 64 ± 6% at 10 years; those operated upon the second decade, 76 ± 6% were event free at 5 years. The incidence of freedom from an anticoagulation complication in the first decade was 89 ± 4% at 5 years and 80 ± 5% at 10 years; for those in the second, 87 1 5% at 5 years.

951

Project Description: Hospital records were reveiwed of 195 patients having isolated congenital or acquired AS with less than 1+ aortic insufficiency, undergoing AVR during decade I (1962-1971) and decade II (1972-1981). Patients with concomitant CAD were not excluded and did not undergo simultaneous revascularization in the second decade unless a left main lesion was present. Patients were more commonly male, 68% and 76% for congenital and acquired AS lesions respectively. The mean age at operation was 58.6 during the second decade versus 49.9 during the first.

The mean peak systolic gradient (PSG) was 50-100 mm Hg in 58%, $>$100 mm Hg in 27%, and $<$ 50 mm Hg in only 4% in all patients studied. Of the 116 (59%) patients having complete hemodynamic studies, the mean PSG was 100 mm Hg, cardiac output/index 5.9/2.8, and calculated aortic valve area/index 0.6/0.3 in the first decade and 83, 5.0/2.7, and 0.6/0.3 respectively in the second decade.

The non-cloth covered Starr-Edwards prostheses were implanted in 68 (34.9%) patients and the cloth covered series in 69 (35.4%) patients. Bjork-Shiley prostheses were used in 28 (14.4%) and Hancock glutaraldehyde-preserved porcine valve bioprosthesis in 25 (12.8%) of patients. Anticoagulation with sodium warfarin was used indefinitely in patients with non-cloth covered Starr-Edwards and Bjork-Shiley prostheses and for six months in patients with cloth covered Starr-Edwards with the exception of the 2400 series. Patients with Hancock bioprosthesiswere not anticoagulated.

Analyses did not reveal significant differences in patients with acquired versus congenital AS relative to operative mortality, long-term survival or complications. Operative mortality ($\leq$ 30 days) was 16% the first decade and 3.4% the second, death being related to hemorrhage, arrhythmia, prosthetic malfunction or congestive heart failure in 80% of patients. In the surviving 175 patients cummulative followup was 995 years. Eight-nine (93.3%) of patients in decade I were functional class (NYHA) I or II at 6 months. Similar results (95.8%) were found in the second decade. Twenty-nine (16.6%) of the surviving 175 patients were lost to followup or not studied.

Postoperative hemodynamic results revealed similar pressure drops across the various valves used with similar CO/CI measured, with the exception of the 2300 series Starr-Edwards prosthesis. Four patients required re-operation with the 2300 series valves because of PSG ranging from 46 to 114 mm Hg.

Ventricular function was difficult to evaluate in these patients and the use of left ventricular end-diastolic pressure (LVEDP) was not as useful as left atrial (LA) or pulmonary arterial wedge (PAW) in assessment of LV function, but was more uniformly measured. Recognizing that the LVEDP was not an independent indicator of LV function, 39.5% of all patients improved to normal function; 32.8% had LV function preserved; 18.5% remained abnormal; and 9.2% had damage to the myocardium following operation.

Late mortality occurred in 66 patients, 47 operated upon in the first decade and 19 in the second. Death occurred at a mean of 53.4 and 37.8 months after operation in the first and second decade respectively. Causes of death were similar to those occurring early and included hemorrhage, arrhythmias, prosthetic

malfunction, congestive heart failure, and myocardial infarction accounting for 55% and 42% during the two decades. Death was attributed to an embolus in 4 patients.

Symptomatic evaluation of surviving patients, including those lost to followup (n=29), at the time they were last seen, revealed 83% of patients operated upon the first decade and 95% in the second continued to be functional class I or II at a mean followup of 126.8 and 43.1 months for the two decades. Only 10% of patients were functional class III and none were IV.

Actuarial survival revealed a 5 year survival of 65.8 ± 5% and 71.7 ± 6% for patients operated on the first and second decade respectively and 59.1 ± 5% at 10 years for first decade patients. Thus, the "improved prostheses", better operative and myocardial protection techniques and earlier operative intervention altered the operative mortality but not the long-term survival.

Thromboembolism and anticoagulation complications, primarily bleeding, related to chronic use of sodium warfarin were the most frequent clinical events following AVR. Of patients operated upon the first decade 73 ± 5% were event free of thromboembolism at 5 years and 64 ± 6% at 10 years; those operated upon the second decade, 76 ± 6% were event free at 5 years. More than one thromboembolus occurred in 9 (20.5%) of 44 patients (mean 3.1 emboli per patient). Late death was attriubted to thromboembolism in 4 patients. Of the 137 Starr-Edwards valves implanted, 22% of patients with non-cloth covered valves had an embolus at an average of 54.5 months and 35.4% with cloth covered valves had one at an average of 39.3 months.

The incidence of freedom from an anticoagulation complication for patients undergoing AVR in the first decade was 89 ± 4% at 5 years and 80 ± 5% at 10 years for those in the second, 87 ± 5% at 5 years.

Proposed Course: Study completed

Publication:

McIntosh CL: Operative treatment of adults with aortic valve stenosis: results and factors influencing the results. In: Aortic Valve Stenosis and Aortic Regurgitation (Ed. W.C. Roberts). Yorke Medical Books, In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02735-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mitral valve replacement with and without chordal excision

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI
Michael Jones, Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Surgery Branch

SECTION:

INSTITUTE AND LOCATION: National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS.	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

xx ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

xxx ☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this clinical trial is to test the hypothesis that the mitral valve apparatus (leaflet, chordae tendineae and papillary muscles), if left intact at the time of valve replacement, will prevent the nearly uniform postreperfusion left ventricular dilation and low cardiac output associated with surgical treatment of mitral insufficiency of long duration.

The specific aims are: (1) measure LV hemodynamics and dimensions with ultrasound prior to and after cardiopulmonary bypass in the OR; (2) replace the mitral valve in patients with pure mitral regurgitation with and without resection of the entire mitral valve apparatus; (3) determine immediate postoperative hemodynamic characteristics of each group; (4) analyze 6 month and 3 year results in terms of exercise capacity, LV dimensions and hemodynamic criteria.

The rationale of this investigation is the hope that if the immediate postbypass course of patients receiving mitral valve replacement for long standing mitral insufficiency can be altered by the mechanism of preventing left ventricular dilation by maintaining the innate physical structures of the left heart, operative mortality and support measures may be reduced and long-term benefits may accrue.

The results in four patients have been excellent in three and no change in the expected left ventricular failure in one patient. Two patients have had bioprostheses and 2 patients have had ST. Jude prostheses. Matched paired patients having mitral valve replacement during the same interval have had subtle changes of left ventricular dysfunction in the postoperative interval which appear typical and different than the experimental group. Six month data will be obtained soon.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description: This clinical trial tests the hypothesis that the chordal-papillary muscle annular ring and leaflet apparatus is important in left ventricular mechanics associated with mitral insufficiency. The evidence for this is divergent and subtle dating back to studies by Lillehei in 1960. Low cardiac output was associated more often when the papillary muscles were excised than when these structures were left intact. Animal data suggests that in normal ventricles, excision of the entire valvular apparatus and mitral valve replacement results in an increase of 20-40% systolic and diastolic dimensions at normal to low preload. A clinical trial in Hannover, West Germany and Toronto, Canada show similar good results by maintaining continuity of these structures.

Mitral valve replacement in patients is performed in standard manner. The only exception is that little if any of the mitral leaflets tissue and/or chordae tendineae are resected. The suture techniques are identical whether or not the valve is removed. Pressure, flow and ultrasonic studies are used before, and after valve replacement with the chest open. Hemodynamic studies are performed in the intensive care unit for 24-72 hours. Six months after surgery, the patients are to be restudied by cardiac catheterization, nuclide angiography, and ultrasonic studies. Four patients have had this procedure; two having a St. Jude bileaflet mechanical prosthesis and two a Hancock bioprosthesis. The results in three patients have been excellent with little to no ventricular dilation, increase in cardiac output, and no requirement for any type of catecholamine therapy. One patient with IHSS has had the expected low output course. Four patients, matched for age, sex, and extent of disease have all had subtle difficulties after mitral valve replacement for mitral insufficiency with complete excision of the valve.

Proposed Course: The study is to continue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02736-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The clinical and hemodynamic results of triple valve replacement at the NIH.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Warren M. Glover, M.D., Clinical Associate, Surgery Branch, NHLBI

Edward Lakatos, Ph.D., Statistician, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Gail Greenberg, Computer Technologist, NHLBI
Pat Strobel, Computer Technologist, NHLBI

COOPERATING UNITS (if any)

Division of Heart and Vascular Diseases, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	0.75	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A retrospective analysis of 54 patients undergoing concomitant aortic, mitral and tricuspid valve replacement at the NIH was performed. Pre and postoperative catheterization data was subjected to statistical analysis as well as patient age, patient sex, history of prior cardiac surgery and date of operation. Patients were grouped by valve type to assess differences in long-term morbidity and mortality. Computer generated cumulative survival and event-free survival curves were compared. Findings included the following: 1) Operative survivors had a significantly lower preoperative mean pulmonary artery pressure, 2) decrease in pulmonary artery systolic pressure following operation had a positive correlation to long term survival, 3) advanced age had a significant influence on operative mortality but patient sex and history of prior cardiac surgery did not, 4) patients with all porcine valves had an improved raw survival and event-free survival compared with other combinations of prosthetic valves.

956

Project Description: A retrospective analysis of 54 patients undergoing concomitant aortic, mitral, and tricuspid valve replacement at the National Institutes of Health (NIH) was performed. All patients requiring triple valve replacement (TVR) from July 1, 1966 to September 1, 1983, were included. Preoperative cardiac catheterization data were available for 53 of the 54 patients. Following TVR, all surviving patients, with one exception, underwent cardiac catheterization six months to one year from the date of surgery. The mean follow-up time for survivors was 60 months. The early (30-day) operative mortality was 15 of 54 (28%). Thirty-seven survivors were followed yearly at the NIH with 14 late deaths. All deaths were cardiac in origin. The five-year survival was 59% ± 9%, while the five-year event-free survival was 40% ± 8%. Age had a significant influence on operative mortality. However, patient sex and a history of prior cardiac surgery did not. Operative survivors had a significantly lower preoperative pulmonary artery (PA) mean pressure (38 ± 4 mm Hg) compared to operative deaths (55 ± 6 mm Hg), $p \leq .005$. The average decrease in PA systolic pressure after operation was 18 ± 2 mm Hg, which had a positive correlation with long-term survival based on an analysis using the Cox model, life table analysis, $p \leq .03$. Patients were grouped according to valve type; 9 patients had three mechanical valves (Group I), 13 patients had three porcine bioprosthetic valves (Group II), and 32 patients had mixed porcine and mechanical valves (Group III). Survival curves and event-free survival curves for the three groups were analyzed. The all porcine group (Group II) appeared to have an improved survival and event-free survival compared with other combinations of prosthetic valves although this finding did not reach statistical significance ($p = .09$ and .06, respectively).

Proposed course: Paper to be submitted to the Journal of Thoracic and Cardio-Vascular Surgery.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02737-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Tricuspid valve disease associated with pure mitral regurgitation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Steven R. Cohen, M.D., Clinical Associate, Surgery Branch, NHLBI

Jeffrey E. Sell, M.D., Clinical Associate, Surgery Branch, NHLBI
Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Surgery Branch

SECTION:

INSTITUTE AND LOCATION: National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [x] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tricuspid valve disease has generally been dealt with as an isolated valvular lesion. However, tricuspid regurgitation generally occurs in association with mitral valve disease. Tricuspid regurgitation results from either organic disease of the valve or more commonly from the effects of mitral valve disease of the right ventricle and the tricuspid valve annulus.

To determine the incidence and severity of tricuspid disease in association with initial mitral valve disease we studied a group of 47 patients with pure, chronic mitral regurgitation with and without tricuspid valve regurgitation. Preoperative features and operative findings were statistically compared. The 22 patients without and the 25 patients with tricuspid regurgitation were similar except for the duration of symptoms. The latter group had a six year or more duration whereas those without tricuspid regurgitation came to surgical palliation much sooner. Palliation of symptoms was of longer duration in those without tricuspid regurgitation. These data suggest that postponment of mitral valve surgery after the onset of significant symptoms have occurred results in poor prognosis.

95-8

Project Description: Forty-seven patients with chronic, pure mitral regurgitation were studied retrospectively. Twenty-two patients (47%) had no tricuspid regurgitation preoperatively, whereas 25 patients (53%) had tricuspid regurgitation by clinical history and physical examination that was confirmed at operation. Preoperatively, there was no significant difference between the two groups in terms of functional class, age, sex, severity of symptoms, or hemodynamic findings. Patients with duration of significant symptoms greater than six years had a statistically higher incidence of tricuspid regurgitation than those with less duration of symptoms. Although jugular venous distention, ascites and peripheral edema were not associated strictly with tricuspid regurgitation, a systolic murmur increasing with inspiration, an enlarged pulsatile liver, a tender liver, and a positive hepatojugular reflux were statistically more common in patients with tricuspid regurgitation than in those without. Long-term followup has shown that those patients with tricuspid regurgitation fare less well than those who have had a surgical procedure performed on the mitral valve earlier in the course of the disease. These data suggest that earlier palliation of mitral regurgitation may prevent tricuspid regurgitation and provide improved long-term palliation.

Proposed Course: The analyses of long-term followup will be completed and a manuscript submitted for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02738-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Degenerative changes in tricuspid and mitral porcine bioprosthesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Steven R. Cohen, M.D., Clinical Associate, Surgery Branch, NHLBI

Marc A. Silver, M.D., Clinical Associate, Pathology Branch, NHLBI
Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Late degenerative changes in simultaneously implanted and explanted tricuspid and mitral porcine (Hancock) bioprostheses have never been compared. Little information is available concerning degenerative changes in porcine bioprostheses in the tricuspid position implanted in humans. Previously, we compared morphologic alterations in simultaneously implanted and explanted porcine bioprostheses in the aortic and mitral positions. Aortic bioprostheses degenerated at a less rapid rate than the corresponding mitral bioprostheses. The present study examined the relative rates of degeneration of bioprostheses in the tricuspid and mitral positions. Six patients had pairs of valves implanted and explanted simultaneously at a mean of 3.75 yrs. The major findings were degenerative changes in all 12 valves examined with an accelerated rate for bioprostheses in the mitral position.

960

Project Description: Accordingly, 12 PBs (6T and 6M) from 6 patients, 2 men and 4 women, aged 30 to 64 years at implantation (mean 45) were re-examined 62 to 128 months (mean 100) later. Anatomic degenerative changes were present in all 12 PBs. Calcific deposits were more extensive in the M PBs in 3 of 6 pairs, greater in the T PBs in 1 of 6 pairs and equally distributed in 1 of 6 pairs. The most extensive calcific deposits were in the 1 patient aged 30 years at implantation. Cuspal tears were present in 5 PBs, all from the M position. Thrombus was present in only 1 PB from the T position. Fibrous thickening was present in 4 pairs of PBs and equally distributed between the M and the T PBs. Thus, anatomic degenerative changes occur in both T and M PBs implanted simultaneously, however, degeneration is most severe in the PBs explanted from the M position.

Proposed Course: Study completed

Presentation:

American College of Cardiology, 33rd Annual Meeting, Dallas, TX, March 1984 (S.R. Cohen)

Publications:

Cohen SR, Silver MA, McIntosh CL, and Roberts WC: Comparison of late (62 to 120 months) degenerative changes in simultaneously implanted and explanted porcine (Hancock) bioprostheses in the tricuspid and mitral valve positions in six patients. Am J Cardiol 53:1599-1602, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02739-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prosthetic Heart Valves: A pictorial summary

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation.)*

Steven R. Cohen, M.D., Clinical Associate, Surgery Branch, NHLBI

Lawrence R. Glassman, M.D., Clinical Associate, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The radiographic features of commercially available prosthetic heart valves and the clinical aspects are summarized on a series of posters. As patients present in congestive heart failure years after a valve replacement at a hospital unfamiliar with the patient's history, posters such as ours would be available to check for the exact valve whether in the aortic or mitral position. Our poster will also enable the clinician to quickly determine reported valve durability, hydraulic performance, thromboembolic rates, degree of hemolysis and overall characteristics.

962

Project Description: Approximately 80 photographs and radiographs were obtained either from David Mehlman, M.D., Division of Cardiology, Northwestern University Medical Center or from Surgery Branch, NHLBI resources. The photographs were linked with the appropriate radiographs and several posters are planned. A poster of Aortic Valves, Tissue and Mechanical; a poster of Mitral Valves, Tissue and Mechanical (Cage-Ball, Cage-Disc and Tilting Disc) will be the first two posters to be completed.

Proposed Course: Continuation of program as new heart valves are placed in clinical use.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02740-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Coronary vascular tone after coronary bypass operations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Artwohl, M.D., Clinical Associate, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard Cannon, M.D., Senior Investigator, Cardiology Branch, NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This clinical study tests the hypothesis that coronary blood flow dynamics of patients may be altered in the immediate interval after a coronary artery bypass procedure which may be deleterious to the patient's future course if unrecognized. Six patients have had complete studies. A thermal dilution catheter is placed into the coronary sinus and threaded to the great anterior vein. Ports are at the tip and more proximal for mixed effluent sampling. Blood samples are used to determine regional oxygen consumption, acid base balance, lactic acid, pyruvate, creatine kinase and lactic dehydrogenase isoenzyme concentrations. Complete hemodynamic evaluations are performed in serial fashion for 6-8 hours after operation. The results to date show wide variation of response to blood flow augmentation. In some, no significant changes in coronary vascular resistance, cardiac output, or any of the biochemical variables occur. Other patients show changes which can be related to less than optimal contractility and systemic flow. One patient demonstrated an increase in coronary vascular resistance prior to a decrease in cardiac output. This was reversed with a calcium channel blocking agent. The project continues.

964

Project Description: Adult males greater than 40 and less than 70 years of age who require more than one coronary artery bypass graft were selected. Informed consent is obtained. Prior to the induction of anesthesia, appropriate catheters are inserted into the left radial artery, superior vena cava, pulmonary artery, and coronary sinus. The last is advanced to the level of the great anterior cardiac vein which parallels the left anterior descending coronary artery. Complete studies are obtained in the catheterization laboratory, after induction of anesthesia, prior to and after cardiopulmonary bypass, and every hour thereafter for six to eight hours. Regional oxygen consumption, pH, pCO_2, pO_2, arterial and venous saturations, coronary and systemic blood flow, lactate, pyruvate, creatine kinase and lactic dehydrogenase isoenzyme concentrations are determined by serial sampling of various blood pools. Systemic, pulmonary, and coronary artery resistances are calculated as are left and right ventricular stroke work. Data analysis consists of time-concentration plots of the major variables.

Proposed Course: This study is to continue until 15-25 patients have had complete data.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02741-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Coronary artery bypass procedure for severe disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Jose Montalvo III, M.D., Clinical Associate, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This is a retrospective study examining the intermediate results (five years) of CABG on patients who would be considered at "high risk" because of pre-operative factors such as low ejection fraction, poor coronary artery anatomy, poor results on noninvasive evaluation, etc.

This is certainly a population which is being seen more frequently by the cardiac surgeon and an assessment should be made as to whether these people benefit from this operation.

966

Project Description: There are 39 patients who have undergone CABG from 1978 to June 1983 who could be considered at "high risk" because of poor left ventricular function and depressed ejection fraction ($< 30\%$) (normal $58 \pm 5\%$). This group has shown an operative mortality rate of $=11\%$ and a six month graft patency rate of $=74\%$ of those grafts studied.

At this time we are assessing the intermediate symptomatic and functional palliative worth of the operation for these patients. Some patients have not benefited from their operation. Various preoperative parameters are under analysis to determine predictive values. These include noninvasive tests such as exercise tolerance test, radionuclide angiography, electrocardiogram as well as cardiac catheterization data and coronary anatomy as judged by measurements from the selective coronary angiograms.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02742-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assessment and use of new ultrasonic technologies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

Elling Eidbo, B.A., Research Assistant, Surgery Branch, NHLBI
Lilliam M. Valdes-Cruz, M.D., Guest Worker, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	3	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

New modes of Doppler ultrasound and new signal conditioning of the received sonic spectrum permit accurate assessment of mean and peak velocities of blood flow. New systems will, in the near future, permit quantitation of the entire flow field velocities. Three studies using these technologies have been completed. (1) quantification of obstruction and regurgitation in right ventricular to pulmonary artery conduits with and without prosthetic valves; (2) quantification of aortic insufficiency; and (3) correlation of pressure drops across stenotic bioprosthetic valves in the mitral and tricuspid position. The results show that high correlations of pressure drop by Doppler measurements to those measured simultaneous by traditional methods. Contrast echocardiography was successful in quantitation of aortic insufficiency. This noninvasive system has been used to determine the degree of regurgitation and obstruction in two patients to date.

96F

Project Description: The development of continuous wave Doppler systems with multiple frequencies together with pulsed, 2-D and M modes in one device has opened a new field of noninvasive accurate quantitation of a variety of pathophysiologic hemodynamic conditions. Additionally, this technology may permit serial assessment of the palliative benefits or lack thereof in a host of operations for congenital and acquired heart disease. The benefits of continued exploration of these technologies are (1) accurate noninvasive determination of improvement or deterioration of diseased native or prosthetic heart valves and conduits; (2) avoidance of repeated cardiac catheterizations; and (3) more frequent observation in the outpatient setting as opposed to costly hospitalization. The three projects illustrate the rapidly expanding field of use for these technologies.

Twenty-nine RV to PA conduits (23 with and 6 without valves) were implanted in juvenile baboons for 12 months. The PA had been transected and oversewn proximally. Catheterizations and angiographic, ultrasonic and morphological studies were performed. Angiography performed in the lateral view demonstrated progressive narrowing of the lumenal area of the conduit and estimated regurgitation. Simultaneous distal PA and RV pressures and synchronous continuous and/or pulsed wave Doppler velocities were recorded. Doppler interrogation was performed with 2-D echocardiographic guidance and audio steering (IREX scanner) to obtain maximal velocity signals (0.7-5.2 M/sec). Fourteen ultrasound studies were obtained using right parasternal and subcostal short axis views. Pressure drops were calculated: $4 \times (\text{maximal velocity})^2$.

	RANGE (mm Hg)	MEAN (mm Hg)	r	p value
Cath ΔP	13 - 110	42 ± 10	0.94	0.001
Doppler ΔP	2 - 107	35 ± 9		

Doppler velocities predicted conduit obstruction by the relation Cath ΔP = 0.99 (Doppler ΔP) + 7.2. The ultrasonic diastolic spectral velocity profiles qualitatively estimated the severity of conduit regurgitation as determined by pulmonary angiograms. At necropsy, the morphologic pathology of the conduits was identical to that found in patients. Thick fibrous peels proximal to the valves produced the obstruction. Valve cuspal fusion to the conduit caused the regurgitation. Calcification of the valves was not a prominent feature. These data demonstrated that new noninvasive ultrasonic techniques can predict the severity of RV to PA conduit lumenal obstruction and evaluate conduit regurgitation. It is concluded that this technique will be useful in following children with RV outflow tract conduits and aid in intervention prior to the development of RV dysfunction.

In the aortic insufficiency (AI) study, AI was produced acutely in 3, 25Kg open chest lambs by resecting an aortic valve leaflet in order to explore contrast echo quantitation of regurgitation. Calibrated electromagnetic (EM) flow meters were placed on the pulmonary artery (PA) to measure effective cardiac output (CO), and on the ascending aorta (AO) to determine total forward AO flow (FF). The difference between FF and CO=reverse AO flow/min, so that regurgitant fraction (RF) could be calculated. RF was varied with a Neosynepherine drip to yield 22 EM RFs 14-58%(CO 1.3-3.1 l/min). 2D echos were recorded with an IREX

scanner in apical views to image the AO with constant time gain compensation and instrument gain. Left atrial injections of 0.25cc (75 mgs) of SH U 454, an experimental gas producing echo contrast agent (Berliscan, Inc.), filled the left ventricle and AO uniformly (202-220 video density (V-DENS) units). AO time V-DENS dilution measurements were fitted to exponential curves. The exponential V-DENS decay constant, N/heart rate, showed a linear relationship ($r = -.84$, $p < 0.001$ to EM RF. The time to ½ of peak AO V-DENS/RR' interval was also correlated (r=0.81) with RF. Our study suggests that V-DENS studies with a standardized echo contrast agent may allow quantification of valvular insufficiency when CO is normal.

We performed 2D Doppler echo exams on 22 sheep previously implanted with bovine pericardial or porcine aortic valves in mitral (MV) (n=9) or tricuspid (TV) (n=10) positions. Doppler exams, using an IREX scanner and simultaneous hemodynamic studies were performed a mean of 4.5 months post-op and just prior to sacrifice. Doppler velocities (VEL) at rest and after Isuprel were corrected for the angle between sampling and flow. Data did not differ between valve types or locations, so results were combined. Peak diastolic VEL (n=45) between 120-290cm/sec correlated well with peak gradients between 6-43mm Hg (r=0.90, SEE=16 cm/sec). Using the simplified Bernoulli equation (gradient= $4 \times \text{Max VEL}^2$) Doppler VEL predicted actual peak gradients well (r=0.89, SEE=2.6mmHg). Doppler mean diastolic VEL, however,predicted actual mean gradients poorly (r= 0.56 SEE=2.1mm Hg). Peak VEL and gradients occurred late in diastole, so pressure half time could not be determined, but Gorlin valve areas ranging from 0.31-2.87cm^2 were predictable with wide scatter by the formula A=0.003 x Heart rate x Cardiac output/mean VEL (r=0.73, SEE=0.3cm^2). Valve specimens showed leaflet thickening and calcification similar to degenerated bioprostheses from patients. The Doppler method, while predicting valve area only marginally, appears promising for predicting gradients across bioprosthetic TV's and MV's.

Proposed Course: These projects will continue and new uses explored to make possible clinical assessment of a wide range of cardiac abnormalities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02743-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The development of a specific immune tolerance model in rhesus monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert D. Moses, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Ronald E. Gress, M.D., Senior Investigator, Immunology Branch, NCI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
David H. Sachs, M.D., Chief, Immunology Branch, NCI
Eli Glatstein, M.D., Chief, Radiation Oncology Branch, NCI
Martin L. Morin, D.V.M., Chief, Primate Research Unit, DRS

COOPERATING UNITS *(if any)*

Immunology Branch, NCI
Radiation Oncology Branch, NCI

LAB/BRANCH: Surgery Branch

SECTION:

INSTITUTE AND LOCATION: National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project is structured to test the hypothesis that immunologically mature rhesus monkeys transplanted with a genetically-mismatched heterotopic heart allograft at a time when the recipients' immune systems have been rendered incompetent with myeloblative total body irradiation will develop specific and permanent tolerance to the allografts. The hypothesis is based on the clonal selection theory of Burnet, confirmed experimentally by Medawar, which states that immune-competent lymphocytes present during fetal life which are reactive with self-antigens are permanently inactivated or eliminated. Myeloablative total body irradiation followed by autologous marrow transplantation after lymphocyte depletion of the marrow is the method employed to recreate a fetal immune environment in immunologically mature animals. Much effort has been devoted to the development of a working irradiated-monkey model and lymphocyte depletion of bone marrow in preparation for the experiments. Nearly all difficulties with these methods have been overcome, and experimentation is to begin soon.

971

Project Description: Much effort has been devoted to achieve uniform survival of total body irradiated, autologous marrow transplanted rhesus monkeys. Most of the initial studies were unsuccessful, primarily because of complications of gut toxicity, but also because of sequelae of marrow suppression. Improvements in post-irradiation care, including the use of nasogastric free water, high-dose potassium, and broad-spectrum nonabsorbable antibiotics, liquid diet supplements when gut function returns to normal, and intravenous dextrose-lactated Ringers infusions, have resulted in nearly 100% animal survival (5 of the most recent 6). The lymphocyte depletions of the bone marrow are to be performed with complement-mediated lysis in the presence of a panel of mouse anti-human lymphocyte monoclonal antibodies known to cross-react with rhesus lymphocytes. The primary antibody in the initial assays, "9.6", was discovered recently to react poorly with fresh rhesus lymphocytes, although the initial assays, all performed with frozen then thawed cells, showed excellent activity. A recent screening has demonstrated that antibody "22" developed in the Immunology Branch may be an acceptable substitute as a pan-T lymphocyte antibody, and lysis assays are underway. The three other T lymphocyte-subtype antibodies, "9.3", "51.1", and "66.1", show excellent activity. Heterotopic heart transplants have been performed in dogs, monkeys, and cats, and technical problems encountered with the early procedures have been solved. Two animals have undergone total body irradiation, autolgous marrow transplantation, and a sham heterotopic heart transplant. One animal succumbed from an operative complication; the other is alive and well one month postoperatively. RhLA typing of the rhesus monkey colony is to take place in the near future. Finally, with successful completion of preliminary studies noted above, the experimentation is anticipated to begin soon.

Proposed Course: The project will be continued over the next 12 months.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02744-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intramyocardial pressure: effect of Ca blockers on regional response.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey E. Sell, M.D., Clinical Associate, Surgery Branch, NHLBI

Randolph E. Patterson, M.D., Chief, Section of Experimental Physiology and Pharmacology, Cardiology Branch

George Alfieris, B.S., Medical Technician, Section of Experimental Physiology and Pharmacology, Cardiology Branch

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Consecutive occlusions of foxhound left anterior descending coronary arteries were performed to study effects of diltiazem (D), and Verapamil (V) on regional cardiac function in response to ischemia. Blood flow, ATP, lactate and fractional shortening were assessed for ischemic and non-ischemic regions. Intramyocardial pressure was measured using millar microtransducers, and compared to the more conventional measurements to assess its value as a tool for studying regional cardiac function.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description: The left anterior descending (LAD) coronary artery of foxhound dogs was isolated via a left thoracotomy under chloralose general anesthesia. An aneroid constrictor was applied, and a region served by this artery selected as the ischemic region. A region served by the left circumflex coronary artery was used as the nonischemic control region. Dogs were put into an experimental group on a rotating basis, the three groups being control (C), Diltiazem (D) and Verapamil (V). Aortic and left ventricular pressures were monitored with Millar microtransducers inserted via the left femoral artery and carotid artery. An LA line was used for injection of 15 micron radiolabeled microspheres. Millar microtransducers were inserted into the myocardium of the ischemic and non-ischemic regions to a depth of 10 mm for measurement of intramyocardial pressure (IMP). Pairs of ultrasonic microcrystals were placed at a depth of 10 mm and a separation of approximately 15 mm on the ischemic and nonischemic regions to measure contractility and fractional shortening. Once the monitoring equipment was affixed and stable, an occlusion was performed lasting 2.5 minutes. After 1 minute, 15 micron microspheres radiolabeled with Cesium were injected to determine myocardial blood flow. After the occlusion was released, the hemodynamic variables were allowed to return to baseline. At least one half hour was allowed between occlusions. If the animal was to receive a drug, this was begun after the hemodynamics were stable, and the dose adjusted to lower the mean aortic pressure by 10%. Control dogs received a comparable saline infusion. A second and final occlusion was performed when the animal was stable on its drug infusion, this one lasting three minutes. After one minute, 15 micron spheres labeled with Scandium were injected. At the three minute mark, 1 cm diameter full thickness biopsies were cored, and immediately frozen in liquid nitrogen for assessment of ATP and lactate levels. The heart was then removed and cut for assessment of regional blood flow.

Results: Diltiazem and Verapamil significantly preserved ATP in the ischemic regions to the level of the nonischemic region during these short occlusions. Control animals showed a significant drop in ATP. Lactate levels also rose insignificantly in the diltiazem and verapamil animals, but rose significantly in controls. Flow to the ischemic region was significantly less than to the nonischemic region in all cases, being less than 10% normal flow. Neither drug appears to affect ischemic region flow. Analysis of hemodynamic data is incomplete at this time, but preliminary analysis seems to show a greater drop in contractility both by fractional shortening and by generated pressure in animals receiving calcium channel blockers than in control.

Proposed course: Analysis of hemodynamic data and flow data will be completed and publication is planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02745-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Relation of pH during ischemia to myocardial recovery.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Greg H. Ribakove, M.D., Guest Worker, Surgery Branch, NHLBI

Lawrence R. Glassman, M.D., Clinical Associate, Surgery Branch, NHLBI
Thomas J. Takach, M.D. Clinical Associate, Surgery Branch, NHLBI
James Voigtlander, B.A., Laboratory Technician, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

Laboratory Animal Medicine and Surgery, NHLBI

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We studied the effects of global normothermic myocardial ischemia on myocardial pH changes. The predictability of recovery of function in sheep following global ischemia is enhanced when pH is preserved at 7.0 or greater. Continuous monitoring of myocardial pH was a reliable predictor of recovery of left ventricular stroke work.

975

Project Description: The interrelationships of myocardial pH to recovery of left ventricular function after normothermic global ischemia was studied in 20 sheep. pH was measured with a new fiberoptic probe mounted within a 23 gauge needle. The probe consists of two single strand optical fibers which transmit changes in the color of the pH indicator phenol red. After a left thoracotomy, arterial, left atrial, left ventricular and pulmonary catheters and multiple intramyocardial pH and temperature probes were placed. Stroke work (SW) versus left atrial pressure (LAP) curves were generated by volume loading with whole blood to LAP of 5-15 mm Hg. Preischemic myocardial pH was 7.42 ± 0.02. Control animals (n=5) underwent one hour of cardiopulmonary bypass (CPB) without ischemia. Three groups of 5 animals each had the aorta cross-clamped and the pH was allowed to fall to 7.0, 6.8, or 6.5. Myocardial temperatures during global ischemia were similar in all groups. One hour after termination of CPB hemodynamic curves were again determined. The percent recovery of LVSW was derived from the ratio of the integrals of the pre- and postbypass stroke work curves. Data are expressed as mean values ± S.E.M. Groups were compared using the unpaired Students t-test.

pH Group	n	Ischemia Time	% Recovery of LVSW
Control	5	0 min	95 ± 4
7.0	5	10 ± 1 min	86 ± 5
6.8	5	14 ± 1 min	45 ± 4
6.5	5	25 ± 3 min	38 ± 8

There is no statistical difference in recovery of function between control and pH 7.0 groups. The decrease in function in pH 6.8 and 6.5 groups is statistically significant ($p < 0.001$) when compared to pH 7.0 or control groups. Thus, in this animal model system, myocardial function decreases significantly when pH falls below 7.0. Continous fiberoptic measurement of myocardial pH during global ischemia reliably predicted recovery of LV function in animals and may prove to be a useful adjunct in clinical cardiac surgery.

Presentations: To be presented at the American College of Surgeons meeting, October 1984, San Francisco, California.

Publications: "On Line Fiberoptic pH Microprocessor System Predicts Recovery of Function Following Global Myocardial Ischemia". To be published in Surgical Forum, October 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02746-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The effect of hypothermia and cardioplegic solutions on intramyocardial pH

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Thomas J. Takach, M.D., Clinical Associate, Surgery Branch, NHLBI

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Unknown is the effect of hypothermia or various cardioplegic solution compositions at various temperatures on interstitial myocardial pH during global ischemia and reperfusion. Hydrogen ion flux is very rapid in ischemic tissue and especially so during the initial 30-90 seconds of reperfusion. An NIH developed fiberoptic system using membrane captured phenolphthalin can measure pH in tissues continuously although the time constant is long at present. These studies were designed to test the hypothesis that the exponential rate of rise of hydrogen ion concentration in ischemic myocardium is altered substantially by hypothermia because of known pK_a changes in water as a function of temperature. Further, the imidazole component of red blood cell buffer is known to interact but is eliminated in these studies by frequent washout of the coronary system. The preliminary data show a marked blunting of the time - pH decay with hypothermic and cardioplegic solutions.

977

Project Description: Fifty sheep will be used in these studies. After the induction of anesthesia, intubation, volume ventilation, and left thoracotomy arterial, left atrial, left ventricular and pulmonary artery catheters are placed. pH and temperature sensors are placed in the septum and left ventricular free wall. Cardiac output is measured by thermal dilution. Left ventricular stroke work (LVSW) versus mean left atrial pressure ($\overline{LAP}$) curves are generated by volume loading with fresh filtered whole blood to $\overline{LAP}$ of 5-15 mm Hg. The left femoral artery is cannulated with a 14 Fr. cannula and the pulmonary artery with a 28 Fr cannula. Hypothermic cardiopulmonary bypass (CPB) is instituted. A LV vent is placed through the left atrial appendage. Control animals (n=5) undergo one hour of CPB at 24°C without ischemia. Three additional groups of 5 animals each have the aorta crossclamped and the pH allowed to fall to 7.0, 6.8, or 6.5. Myocardial temperature during global ischemia is similar in all groups. Animals are weaned from CPB without inotropic agents. One hour after termination of CPB, left ventricular function curves are again determined. The percent recovery of LVSW is derived from the ratio of the integrals of the pre and postbypass stroke work curves. Other studies involved washout at 15 minute intervals with 150 ml of 28°C buffered plamalyte solution. Additional studies will use the same protocol but will employ cardioplegic solutions of various compositions now in clinical use. The time temperature integral dependency of myocardial recovery will be delineated. Further, the abrupt deleterious changes seen with reperfusion may be altered by various strategies. The preliminary data are shown below.

Hypothermic Modification of Intramyocardial pH

pH Group	N	Ischemic Time	T°	% Recovery LVSW
Control	6	0	24°	91%
7.0	7	24 min	24°	82%
6.8	6	48 min	24°	63%
6.6	3	62 min	24°	24%

Cardioplegic Modification of Intramyocardial pH

Group	pH	N	Ischemic Time	T°	% Recovery LVSW
Normothermic Control *	6.8	5	14 min	38°	45%
Normothermic Cardioplegia	6.8	3	19 min	38°	50%
Hypothermic Control	6.8	5	49 min	24°	63%

* Previous study

To date, the data although preliminary show that hypothermia has marked effects on the -dpH/dt as do various cardioplegic solutions at any temperature. Asymptotic and reperfusion relations have not been characterized. There appears to

9'78

be an inverse relation between interstitial pH and final stable recovery values of myocardium made ischemic and reperfused with diluted autogolous blood.

Proposed Course: These studies will be completed by June 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02747-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Amiodarone dose related myocardial preservation in hypertrophied myocardium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Thomas J. Takach, M.D., Clinical Associate, Surgery Branch, NHLBI

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The hypothesis tested was that amiodarone, a potent cardiotonic agent with unknown mechanisms of action, has a dose related effect on protection of hypertrophied myocardium. The working isolated rat heart preparation was used at 37°C. Filling pressures and afterload were maintained constant before and after 30 minutes of global ischemia. One injection of 12 ml of normal saline only or containing 0.125-12 mg of amiodarone was given at the onset of ischemia. The determinants of recovery were heart rate, aortic flow, and coronary blood flows. The results show a narrow band width dose response with approximately 40% recovery at 0.125 and 0.5 mg dosages. Maximal recovery of 70% of aortic flow was found at 0.25 mg dose. Higher doses produced decremental values of recovery. This study demonstrates that with single dose use at normothermia, amiodarone may be a useful adjunct for cardioplegic solutions but has a narrow dose-response range.

940

Project Description: The purpose of these experiments was to test the efficacy and toxicity of a new investigational cardiotonic agent, amiodarone, for its myocardial protective effects during global ischemia at 37°C. The working isolated rat heart was used to assess dose-response characteristics. Seventy-eight rat hearts from a strain of spontaneously hypertensive rats were used. The hearts were rapidly excised and perfused in Langendorf fashion with oxygenated Krebs-Henseleit solution. After various catheters and transducers were applied, the preparation was converted to the working mode by providing inflow through the left atrium. Aortic flow was measured electromagnetically and the coronary sinus flow by timed volume collections. Temperature, left atrial filling pressure and aortic resistance were maintained constant. After a 15-30 min interval of steady state conditions, all flow was stopped. The aortic outflow cannula clamped, and 12 ml of 37°C normal saline with or without amiodarone was injected into the aortic root in 90 sec. Dosage range was 0.125 to 12 mg. After 30 min the heart was reperfused in retrograde manner and then converted to the working mode. Aortic flow was the primary indicator of recovery. The results are demonstrated in the table below:

Amount of drug in mg in 12 ml 0.9% saline Injectate	N	Recovery	Aortic Flow Preischemic $\bar{x} \pm S\bar{x}$	Aortic Flow Postischemic $\bar{x} \pm S\bar{x}$	Percent Recovery
0	13	4/13	47 ± 2	5 ± 3	10 + +
0.125	9	9/9	45 ± 2	16 ± 4	37 * * + +
0.250	10	10/10	42 ± 2	30 ± 2	70 * *
0.500	13	10/13	46 ± 1	18 ± 4	39 * +
3.0	14	10/14	47 ± 2	20 ± 5	42 * +
6.0	12	9/12	47 ± 2	16 ± 4	34 * +
12.0	7	7/7	46 ± 4	13 ± 3	28 * + +

* $p < 0.05$ and ** $p < 0.01$ vs controls
\+ $p < 0.05$ and H $p < 0.01$ vs 0.25 mg dose

This screening study demonstrates a narrow dose response for amiodarone. Unknown is the effect when added to clinical cardioplegic solutions or under cold conditions. Small doses 125 µg/gm of wet heart weight appear to be protective against global ischemia.

Proposed Course: Studies at 28, 18 and 10°C with normal saline and cardioplegic solutions will be performed. Oxygen consumption, positive and negative left ventricular dP/dt, and myocardial ATP levels will be assessed. If efficacy can be demonstrated, studies simulating clinical conditions will be performed in sheep.

981

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02748-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protective effect of Amiodarone under hypothermic and normothermic conditions.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Thomas J. Takach, M.D., Clinical Associate, Surgery Branch, NHLBI

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Amiodarone is an investigational benzofuran derivative with known antianginal and antiarrhythmic effects. It has been shown to be effective in reducing the size of artificially induced myocardial infarct areas in the canine model and has been theorized but never proven to act in the blockade of the calcium mediated slow channel. This investigation was designed to test the possible protective effect of the drug on the hypertrophied myocardium during global ischemia under normothermic and hypothermic conditions in an isolated working heart model.

Drug treated hearts differed significantly from controls in both normothermic ($p < .001$) and hypothermic ($p < .05$) conditions by enhancing myocardial recovery of function (change in aortic output) after global ischemia. Amiodarone also significantly ($p < .001$) enhanced survivability (restoration of aortic output and heart rate) in the normothermic drug treated group vs. controls. Hypothermic drug treated hearts had significantly ($p < .05$) enhanced recovery of function vs. normothermic drug treated hearts but differences between hypothermic control and normothermic controls were not significant ($p < .10$) indicating possible synergy or addition between hypothermic and drug effects. It is concluded that Amiodarone ameliorated the deleterious effects of global ischemia in hypertrophied rat hearts at normothermic and moderately hypothermic temperatures.

982

Project Description: Hypertrophied hearts from 350 to 425 g spontaneously hypertensive male rats were tested on an isolated, perfused, working heart preparation. In this model, an oxygenated solution of Krebs-Heinseleit buffer enters the left atrium at a standard preload pressure of 10 mm Hg and passes through the left ventricle into the aorta against a standard mean afterload pressure of 80 mm Hg. Coronary sinus flow exits the right heart via the pulmonary artery and is separately collected. After hearts from anesthetized rats were rapidly excised and arrested in buffer solution at 1°C, they were placed on the apparatus and a ten minute period of aortic perfusion with oxygenated buffer at 37° was initiated. Following left atrium cannulation via a pulmonary vein, the hearts were converted to the left atrial perfusion (working) mode for ten minutes and allowed to stablize. Aortic pressures, aortic outputs, heart rates, and coronary sinus outputs were recorded. At ischemia initiation, each heart received a 12 m 37° normothermic (n = 14) or 24° hypothermic (n = 12) aortic root injection of amiodarone (.25 mg) in .9% saline or .9% saline alone. The hypothermic group was allowed to rewarm spontaneously. Hearts were then subjected to 30 minutes of global ischemia within a water jacketed chamber at 37°C followed by 10 minutes of aortic re-perfusion with oxygenated buffer. Following 10 minutes in the working heart mode, hemodynamic determinations were remeasured during steady state conditions.

The unique characteristics of amiodarone as an established antiarrhythmic and possible calcium channel blocker may have significant implications toward potential use of the drug as a cardioplegic ingredient. The results suggest that amiodarone ameliorates the deleterious effects of global ischemia in hypertrophied rat hearts at normothermic and moderately hypothermic temperatures. The protective mechanism of amiodarone may be secondary to 1) calcium channel inhibitor, 2) increased collateral blood supply to myocardium, 3) antiarrhythmic potential or 4) vasodilatory effect of the drug.

Presentations: Protective Effect of Amiodarone During Global Ischemia Under Hypothermic and Normothermic Conditions in Hypertrophied Hearts. American College of Surgeons Meeting, San Francisco, California, October 1984. Thomas J. Takach, M.D., Michael Jones, M.D. and Richard E. Clark, M.D.

Publications: Thomas J. Takach, M.D., Michael Jones, M.D. and Richard E. Clark. Protective Effect of Amiodarone During Global Ischemia Under Hypothermic and Normothermic Conditions in Hypertrophied Hearts. Surgical Forum, In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02749-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development of an angioscopic technique for coronary arteries.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Paul D. Smith, Ph.D., Senior Investigator, BEIB
Howard S. Kruth, M.D., Senior Investigator, Division of Experimental Atherosclerosis
Jim Voigtlander, Technician, Surgery Branch, NHLBI
Rufus C. Seabron, Technician, Division of Experimental Atherosclerosis
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*
Cardiology Branch, NHLBI
BEIB
Division of Experimental Atherosclerosis

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are investigating the use of ultrathin (1.5 - 1.8 mm) fiberoptic catheter systems for direct visualization of intraluminal anatomy in the coronary artery circulation. Using sheep which have been placed on total cardiopulmonary bypass, the aorta is cross clamped and the coronary ostia are cannulated and fine 1.5 - 1.8 mm angioscopes are passed into the right or left coronary arteries.

The coronary arteries are perfusion fixed using a silver chloride to stain endothelium. The amount of endothelial denudation injury is assessed by scanning and optic microscopy. The coronary arteries of 10 sheep have been examined. The results show that major surface improvements to decrease endothelial injury are required. In addition, use of more powerful light sources to adequately visualize proximal coronary artery anatomy have been found necessary.

984

Project Description: Ten sheep have been studied with variable success in visualizing the intraluminal coronary anatomy using an American Edwards 1.5 mm angioscope. Sheep are placed on total cardiopulmonary bypass and the aortic root is crossclamped to create a "bloodless field" by flushing the aortic root with cooled saline (4°C). Recently an Olympus 1.8 mm angioscope which has been acquired has better optics. Comparisons have been made using coupled miniature video cameras to determine resolution, contrast, brightness and depth of field. Two human cadaver hearts have been studied at necropsy. Excellent visualization was obtained in this in vitro setting.

A closed circuit color television system has been constructed for video recording during the passage of the angioscope. The histologic studies of endothelial integrity are in progress.

Proposed Course: Continued animal experimentation until the catheter and video recording systems are perfected. The goal is to perform angioscopy safely as an adjunct to coronary artery bypass operations to assess the integrity of post-endarterectomy vessels as well as to attempt quantitative and qualitative assessment of human atherosclerotic plaque in the coronary arteries.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02750-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of various laser sources on atherosclerotic coronary arteries.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Robert Bonner, Ph.D., Senior Investigator, BEIB
Paul D. Smith, Ph.D., Senior Investigator, BEIB
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
John Deignan, Chief, Laser Laboratory, Goddard Space Flight Center, NASA

COOPERATING UNITS *(if any)*
BEIB
Cardiology Branch, NHLBI
Pathology Branch, NHLBI
NASA: Goddard Space Flight Center
Naval Research Laboratory

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The optimum laser source for performing coronary artery laser angioplasty has not been determined. The purpose of this study is to define the most suitable laser source for vaporization of atherosclerotic plaque. Selected coronary arteries from human cadavers are exposed to lasers of various wave lengths and precise real time measurements of thermal diffusion are made by thermocouples and infra-red photography.

In addition well defined gross and histopathological techniques are used to compare the effects of various laser-tissue interactions. Three laser sources have been tested of which one appears useful for the proposed application.

9F6

Project Description: The coronary arteries from ten patients with atherosclerotic coronary vascular disease are obtained shortly after death. These specimens are then used to test various laser sources in time-energy studies.

Using fast reactive temperature thermocouples on the adventitial surface near the target temperature diffusion gradients are calculated for each wavelength at different energy exposures. Simultaneously, an infrared thermal camera is positioned to record the surface temperature at the laser-tissue interface.

After several exposures using differing energy levels for each laser wavelength these coronary artery specimens are processed for gross and histological evaluation.

In these ways a profile for each laser wavelength is developed enabling us to decide which wavelengths will maximize laser-tissue interaction but also minimize thermal diffusion to surrounding normal tissue (arterial wall).

Thus far three different laser sources have been tested on eight coronary artery specimens. Histological results are pending.

Proposed Course: We intend to use the information from this project to decide which laser source would be most suitable to perform laser angioplasty in an *in vivo* animal model which we are developing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02751-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Laser vaporization of atherosclerotic plaque in swine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Robert F. Bonner, Ph.D., Senior Investigator, BEIB
Paul D. Smith, Senior Investigator, BEIB
John Deignan, Chief, Laser Laboratory, NASA
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI

COOPERATING UNITS *(if any)*

BEIB
Cardiology Branch, NHLBI
NASA - Goddard Space Flight Center
Olympus Optical Company

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

We are creating a colony of atherosclerotic swine with accelerated lesions in the carotid and ilio-femoral arteries. Our intent is to vaporize these atherosclerotic plaques by using laser energy delivered through a fiberoptic delivery system. The animal models are in preparation. Collaboration with scientists at NASA and Olympus Optical Company has been established to develop a new prototype laser angioscope to deliver highly controlled laser energy to atherosclerotic lesions.

Project Description: Twenty swine with atherosclerotic lesions in peripheral arteries (femoral and carotid) will be used. These animals will undergo laser angioplasty using different delivery modes (continuous wave or pulsed) at different wave lengths.

Photochemical pretreatment with Hematoporphyrin Derivative (HPD) to result in fluorescence of atherosclerotic plaque and highly reactive free radicals which will assist in plaque disruption.

The dynamic laser-tissue interface will be measured by sequential arteriograms, eventual sacrifice and histological examination using both light and transmission and electron scanning microscopy.

Various fiberoptic delivery systems, angioscopy catheters, laser wave lengths are under study by *in vitro* experiments.

Proposed Course: Continued development of instrument, animal colony and eventual *in vivo* experiments.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02752-01 SU

PERIOD COVERED: October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Augmentation of vascular supply to ischemic myocardium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jose Montalvo III, M.D., Clinical Associate, Surgery Branch, NHLBI

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to determine the feasibility of augmenting myocardial blood flow by extracardiac methods. Specifically, the use of the internal mammary implant coupled to laser drilled ventricular channels and omental and/or splenic onlay wraps are to be considered. Additionally, the use of endothelial cell seeding together with growth factor may provide further augmentation. The rationale of the proposal is that many patients are not candidates for the coronary artery bypass procedure because of extreme atherosclerosis. There is a possibility that one of the major deficits in long term IHSS patients is ventricular mass/vascular supply disproportion. As an initial step to determine feasibility and develop techniques, forty dogs, in various groups are to be treated with combinations of the internal mammary implant, laser ventriculorophy, omental wrapping, and splenic translocations. The first group has been started. This group is to study the technique developed by Vineberg in Montreal during the decade 1945-1955. Once technically developed, laser techniques now under development will be used to extend the project.

990

Project Description: The project will use large dogs with adequate sized internal mammary arteries. The initial trials will use a left thoracotomy and dissection of the left internal mammary artery (IMA). The heart is exposed, the IMA ligated and transected distally with at least two open side branches. The IMA is tunnelled into the mid myocardium and suture fixed in place. An ameroid constrictor is placed on the anterior descending artery above the first septal perforating vessel. Constriction should take place gradually over a 6 week interval. Additional ameroids may be required in the circumflex system to provide sufficient ischemic stimulus for the development of IMA communication to the sinusoidal system. Additional operative techniques will use free omental grafts obtained by laporotomy and splenic translocation. The latter requires splenectomy with re-establishment of the blood supply by autologous vein bypass grafting techniques. The addition of laser drilling will await determination of the appropriate source, wave length, energy, duration, and mode.

Serial studies will include contrast and nuclear angiography, hemodynamics, and microsphere determination of collateral blood augmentation. Extensive histologic, dye fixation and radiographic studies are planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02753-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
In vivo evaluation of a synthetic trileaflet valve.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Victor Ferrans, M.D., Ph.D., Chief, Ultrastructure Section, Pathology Branch, NHLBI
Elling Eidbo, B.S., Medical Technician, Surgery Branch, NHLBI
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*
Pathology Branch, NHLBI

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)
- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During a thirteen year program, a synthetic trileaflet valve has been developed. Prototype clinical valves use a narrow soft sewing collar of knitted polyester, a flexible coronal shaped stent and a micro-woven fabric which is highly flexible and has stiffness and anisotropic properties similar to normal aortic leaflet tissue. Extensive durability and soft tissue implant studies were performed prior to mitral valve replacement in sheep. The in vivo data showed that valvular insufficiency occurred early (24 hours) which has subsequently been traced to alteration of filament geometry from stress compaction. New fabric designs have been completed and a second prototype is in production.

PHS 6040 (Rev 1/84)

GPO 904-917

Project Description: The purpose of this investigation is to develop a durable trileaflet prosthetic aortic valve made entirely of synthetic materials. The device is to be thrombo-resistant and not require anticoagulants and to have initial antimicrobial properties. Over a thirteen year interval, a device has been developed which has greater than 800 million cycles of function without fatigue. Implant studies of each of the base materials and the finished components show that each has the desired tissue interface reactions. The flexible leaflets have stiffness and anisotropic properties similar to normal human aortic leaflet tissue. Extensive soft tissue studies in rabbits showed no calcification, thickening or biodegradation of the unique composite co-and tripolymeric filaments and yarns. Filaments have ranged from 10 to 23μM with pore sizes of 25-30μM. Spacing of pores was found highly important for the desired blood-material interface reaction.

Through a left thoracotomy, five clinical prototype valves were implanted. Pressure, flow and ultrasonic studies employing M, 2-D, pulsed and continuous doppler modes were used to assess valve function. Prosthetic valvular insufficiency was present in all within 24 hours. Animals were sacrificed at 24 to 10 day intervals. Mitral regurgitation was found to be caused by an alteration of fabric geometry probably by a stress compaction phenomenon. New fabric designs have been completed after implant studies were completed in the soft tissue of sheep. New fabric is in production for a second generation clinical prototype.

Proposed Course: Preimplant in vitro cycling will study adequacy of design and be used to prestress valves for implantation. New soft tissue implants and valve replacements will be performed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02754-01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Blalock-Taussig shunts with prosthetic grafts: long term observations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Jose Montalvo III, M.D., Clinical Associate, Surgery Branch, NHLBI

Robert Artwohl, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypothesis tested in these studies was that prosthetic grafts of specific diameters, biomaterial composition, and lumenal topography have different long term patency rates when used as brachiocephalic to pulmonary artery conduits. The specific aims were to test various types of grafts made from knitted and woven polyester (with and without internal/external velour), microporous expanded polytetrafluroethylene (EPTFE), bovine pericardium or umbilical vein. Fifty-five rhesus monkeys had a left thoracotomy and various grafts (4,5 and 6 mm) with lengths of 2-5 cm inserted between the systemic and pulmonary circulations. There were eight deaths prior to 3 month catheterization. One monkey was unable to be catheterized secondary to poor vessels. Therefore, there were 46 monkeys who underwent 3 month catheterization.

Thus far, there have only been 7 monkeys who have undergone catheterization at one year. Serial angiographic hemodynamic and ultrasonic measurements were made. Preliminary data show that dacron velour has a poor patency (40%) at 3 months. Bovine umbilical vein and bovine pericardium have a patency rate of 71% and 75% at 3 months. EPTFE showed a patency rate of 82% at 3 months.

These data suggest that when prosthetic conduits are used for palliation of pulmonary oligemia in infants and young children, the choice of conduit is highly important for long term palliation. Further, prosthetic materials do not provide the long lasting palliation of the native subclavian artery as described in 1948 by Blalock and Taussig.

994

Project Description: Rhesus monkeys weighing 3-9 kg were premedicated, anesthetized, intubated and volume ventilated. A left thoracotomy was performed. The EKG and arterial pressure were measured simultaneously. In random fashion one of four graft types of any of three diameters (4,5, or 6 mm) was used for construction of a brachiocephalic to left pulmonary conduit using 5-0 polypropylene continuous suture. The chest was closed and the animals were permitted to recover without intervention. Serial angiographic and hemodynamic studies were performed. Injections were made in the aorta to establish patency or occlusion. Pulmonary artery and aortic pressures were measured to determine the pressure drop across the prosthesis. Flow through the conduit was determined by oximetry and measurement of right and left ventricular output. Pulsed and continuous wave doppler studies were performed to determine correlation of noninvasive to invasive pressure drop data and obtain mean and peak velocity through the conduit. Preliminary data are shown below.

Conduit Type & Drain	N	No patent in Mos 3 mos	12 mos	3 month % Patency	12 month % Patency
Bovine pericardium 4,6 mm	12	9	3	75%	25%
Bovine umbilical vein 4 mm	7	5	1	71%	14%
Knitted and woven dacron velour 5 mm	10	4	2	40%	20%
EPTFE - Gortex 4 & 6 mm	17	14	-	82%	-

For some prostheses, failure rate was higher in the smaller diameters. Long-term serial studies continued. Occluded grafts have been submitted for histologic electron microscopic and atomic absorption studies.

Proposed Course: Continuation of serial hemodynamic, angiographic, and ultrasonic examinations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02755- 01 SU

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Afterload reduction in heart failure: effect on blood flow distribution.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Anthony L. Picone, M.D., Clinical Associate, Surgery Branch, NHLBI

Jeffrey E. Sell, M.D., Clinical Associate, Surgery Branch, NHLBI
Randolph E. Patterson, M.D., Chief, Section of Experimental Physiology and Pharmacology, Cardiology Branch

COOPERATING UNITS *(if any)*

Cardiology

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

NHLBI

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.5	1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

After creating Left Ventricular (LV) failure by warm ischemic injury, tissue blood flow in awake sheep was studied using radiolabeled microspheres. Various doses of nipride were used to produce afterload reduction while maintaining left atrial (LA) pressure constant. Pulmonary artery (PA), right atrial (RA), LV, and LA pressures were monitored along with cardiac output (CO).

996

Project Description: Using LV failure model in sheep previously described, LV failure was induced by warm ischemic injury. Approximately 5-7 days post-operatively awake studies were performed. LV, LA, PA, RA, and aortic pressures were monitored using catheters implanted at the initial operation. Thermodilution CO were measured at each experimental stage and 15 micron radiolabeled microspheres were injected to determine blood flow distribution. Animals were studied at base line, and doses of 2, 5, 10, and 20 micrograms per kilogram per minute of nipride. Prior to making measurements, at each drug dose volume in the form of blood or lactated ringers solution was infused to maintain mean LA pressure constant. Following measurements at each drug dose, the nipride was stopped, and the LA pressure allowed to stabilize and a final set of measurements made. The animal was then sacrificed and the organs harvested. Blood flows were calculated for heart, brain, liver, muscle, lung, and kidney tissues.

Results: Cardiac output increased significantly with increasing doses of nipride as expected. Cardiac blood flow to all chambers studied and all layers increased as well. Liver and brain flows also showed increases, as did muscle tissue. Renal blood flow appeared to increase in the cortex, but not to as great an extent as the other organs. Renal Medullary blood flow was essentially unchanged. Hemodynamics appeared to indicate increased cardiac output more in a response to increased heart rate than increased stroke volume and LV stroke work appeared unchanged.

Proposed Course: Indexed and apportioned flow analysis needs to be completed. Publication planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02756-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Myocardial hypertrophy: relation to sensitivity to normothermic global ischemia.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation.)

Jeffrey E. Sell, M.D., Clinical Associate, Surgery Branch, NHLBI

David J. Undershill, M.D., Clinical Associate, Surgery Branch, NHLBI
Lawrence R. Glassman, M.D., Clinical Associate, Surgery Branch, NHLBI
Greg H. Ribakove, M.D., Guest Worker, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

After neonatal banding of either the pulmonary artery (PA) or the aorta neonatal sheep were allowed to grow for one year, producing either left ventricular hypertrophy (LVH) or right ventricular hypertrophy (RVH). Along with a group of control sheep, the experimental sheep underwent a standardized warm ischemic protocol. Intramyocardial pH as monitored by a fiberoptic probe was allowed to fall to 6.8 by cross clamping the aorta during full cardiopulmonary bypass. Left and right ventricular function were measured before and after bypass, and degree of recovery measured by Starling curve creation.

991

Project Description: Neonatal sheep underwent pulmonary artery or aortic banding under general anesthesia during the first 10 days of life. These sheep were allowed to mature for at least one year, resulting in either left ventricular hypertrophy (LVH) or right ventricular hypertrophy (RVH). The sheep and normal controls then underwent a standardized warm ischemia pH evaluation protocol. This consisted of administering general anesthesia using a sodium pentabarbitol drip, and then performing a bilateral thoracotomy. Starling function curves were then constructed by measuring left and right filling pressures, cardiac output (CO) and ventricular afterload (pulmonary artery or aortic pressure). Warm blood was administered to vary the filling pressures to obtain multiple points on the curve. After this was completed, cardiopulmonary bypass was instituted via the femoral artery and right atrium. Once stable on bypass, the pH electrodes were placed in the left and right ventricular myocardium. The aortic crossclamp was applied after venting the LV via the left atrium, and the left ventricular intramyocardial pH allowed to fall to 6.8. The crossclamp was then removed, the pH allowed to return to 7.4, and the animal stabilized. Cardiopulmonary bypass was then weaned, and the animal then underwent a one half hour period of stabilization. Finally, another Starling curve was constructed in the same manner as the first. The animal was then sacrificed by lethal potassium injection. Starling curves were then used to compare degree of recovery of function.

Proposed Course: Study will continue under the direction of Dr. David Underhill.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02757-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of ancrod on nonhuman primates undergoing CPB

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Delwin K. Buckhold, D.V.M., Senior Investigator, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI
Jim Voigtlander, B.A., Technician, Surgery Branch, NHLBI
Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI

COOPERATING UNITS *(if any)*
Cardiology Branch, NHLBI
Hematology Section, Clinical Center

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are investigating the safety of cardiopulmonary bypass in non-human primates which have received the investigational drug ANCROD. The Cardiology Branch has given this drug to patients with recurrent and persistent angina pectoris who are not candidates for surgery. No study to date has shown that these patients can safely undergo cardiopulmonary bypass without severe hemorrhagic complications. A strategy for reversing the defibrinogenating effects of ANCROD has been developed with the Hematology Section.

1000

Project Description: Baboons, weighing 10 - 14 Kg will be injected with ancrod 1.5 units/Kg IV. Within 12 - 24 hours after injection the animal will have a series of hematological tests performed (CBC, PT, PTT, platelet count, FDP, routine chemistry) and be prepared for surgery. The animal is placed on total cardiopulmonary bypass and cooled to 25°C for 45 - 90 minutes and rewarmed. The animal is separated from cardiopulmonary bypass and various regimens used to reverse the effects of both heparin and ancrod. Those animals which develop normal coagulation will be studied for six hours to observe possible rebound phenomena. Whole blood clotting times, activated clotting times, prothrombin time, partial thromboplastin time, fibrinogen and platelet concentration and aggregation characteristics are to be used as criteria of satisfactory reversibility.

Proposed Course: Ten baboons are to be studied in this project with at least one third being controls.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02758-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Creation of accelerated atherosclerotic lesions in swine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI

COOPERATING UNITS *(if any)*
Cardiology Branch, NHLBI

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As part of a project to perform successful laser angioplasty it is necessary to develop a swine colony with severe atherosclerotic vascular lesions within six months. The acceleration is obtained by balloon endothelial denudation of carotid and femoral arteries after which the animals are placed on a cholesterol laden, high fat diet.

Development of vascular lesions will be assessed by ultrasonic, nuclear and angiographic methods. Animals with severe lesions will be used for the angioscopy and laser angioplasty studies.

1002

Project Description: We have secured a colony of 20 Hormel Miniature swine which were to be delivered two months ago but have not arrived yet. They will be placed on a 2% cholesterol diet for one month and then will undergo endothelial balloon denudation with a Forgarty catheter. The vessels denuded will include the carotid arteries and ilio-femoral vessels.

They will then be kept on this "atherogenic diet" for six months with serial cholesterol level measurements and intermittent angiography of selected animals.

Proposed Course: This colony is being raised in order to provide a suitable animal model to study the in vivo effects of laser-tissue interaction and furthermore as a live model on which to perform laser angioplasty. (Please see the review on Laser Vaporization of Atherosclerotic Plaque in Swine.)

Publication: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02759-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pretransfusion platelet morphology and in vivo effect

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Robert Bonner, Ph.D., Sr. Investigator, BEIB
Joseph Fratantoni, Ph.D., Division of Biologics, FDA
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

FDA, Division of Biologics
BEIB

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human platelets demonstrate a wide variety of morphological shapes and sizes. Some believe that this represents a metamorphosis as the platelet passes from discoid shape to round shape to bizarre shape with pseudopod extensions. Others believe this represents a senescence of the platelet and that these forms differ in their potential functional ability to initiate thrombus formation. It is possible that the discoid shaped platelets may be associated with greater adherence, aggregation, and release properties than round or pseudopod shaped platelets.

A new device to noninvasively determine the proportion of discoid-shaped platelets in platelet rich plasma has been developed and standardized. In vivo studies in animals and man involve isotope tagging of pure forms of the animal and human platelet shapes and measuring platelet deposition sites by single and dual radionuclide imaging. Additionally, half life survival times will be determined. This study may lead to a significant improvement in post-operative bleeding syndromes associated with cardiac surgery.

1004

Project Description: The Division of Biologics, Food and Drug Administration, has developed a device which uses the principle of light scattering to determine the percentage of various shapes of platelets in plasma when contained in standard polyvinyl blood bank - FDA approved bags. Quantitation by shape is obtained by direct and differential platelet counts. The hypothesis to be tested is that platelet shape in vitro is related to platelet function in vivo upon re-infusion.

In vitro studies in a flow chamber using high speed photography will determine adherence and aggregation characteristics of pure and mixed populations of platelets. Single and differential isotope tagging will be used to determine site deposition in wounded animals and in cardiopulmonary bypass systems. Finally, similar systems for detection will be used in patients having cardiac surgery to determine aggregation and deposition sites following cardiopulmonary bypass; e.g. heart valves, bypass grafts, wounds.

Proposed Course: The initial in vitro and animal studies will be performed in parallel to acquire sufficient data to submit for application for a clinical trial of differential platelet infusion therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02760-01 SU

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Magnetic targeting of thrombolysin loaded erythrocyte ghosts.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Michael R. Lieber, M.D., Clinical Associate, Pathology Branch, NHLBI
Thomas Clem, Ph.D., Senior Investigator, BEIB
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine and Surgery, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

BEIB
Pathology Branch, NHLBI

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study tests the hypothesis that erythrocyte ghosts can be used to effectively deliver various medications to selected arteries by use of iron laden particles which can be magnetically directed. Further, with a half life of 2-3 days controlled release systems could maintain a therapeutic level of drug without the need for indwelling catheters. The initial trials are designed to deliver thrombolytic agents to specific areas of thrombosis.

1006

Project Description: Thirty rhesus monkeys will be used to create acute thrombi using a copper wire technique in peripheral arteries (femoral, tibial, radial). Concurrently 50 ml of whole blood, will be used to obtain RBC ghosts. The ghosts would be loaded with magnetic particles and the experimental drug. The autologous ghosts are reinfused into the animal while a magnetic field is placed near the artery to be studied. Serial ultrasonic, nuclear and angiographic studies will be used to assess effects of the drug delivery system.

Serum levels of drug will be measured. Control animals will receive infusion of drug and the results compared.

The animals will be sacrificed and liver, spleen and kidney will be examined for the extent of iron deposition. The site of thrombosis will be examined for extent of thrombolysis.

LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1983 to SEPTEMBER 30, 1984

Cardiac and Respiratory Assist Systems

The Section on Pulmonary and Cardiac Assist Devices applied devices to assist the failing heart or the lungs. Such approaches include the design, construction, and evaluation of appropriate blood pumps, artificial lungs, extracorporeal perfusion systems, and blood compatible surfaces.

This year we have explored the effects of mechanical pulmonary ventilation at elevated airway pressures in healthy animals, believing that iatrogenic parenchymal disease develops above some peak airway pressure. We ventilated healthy anesthetized animals and paralyzed animals with a pressure controlled mechanical pulmonary ventilator, monitoring functional residual capacity (FRC), total lung compliance, dead space, cardiovascular hemo-dynamics, and arterial and mixed venous blood gases. All studies were to last 48 hours, or sooner if terminated by severe respiratory failure. A control group of animals ventilated at normal respiratory rates, tidal volumes and airway pressures, showed excellent lung function over the 48 hour study period, and lungs grossly appearing normal throughout at autopsy. Sheep ventilated to an airway pressure of 50 cm H_2O had an initial substantial improvement in all measured variables lasting for the 2-11 hours, only to progressively decline in the following hours. All animals developed severe respiratory failure and most were electively sacrificed due to severe deterioration well before the full 48 hour study period was over.

In studies similar to the above at peak airway pressure of 30 cm H_2O, the course was very similar, except more mild. Most animals survived the full 48 hours study period, although they all manifested severe functional derangement in lung function, with X-ray findings consistent with severe ARDS (adult respiratory distress syndrome).

Our studies were the first to document deleterious long term effects of mechanical pulmonary ventilation on normal lungs, at pressures in excess of 30 cm H_2O. Our results show major parenchymal pulmonary damage, and functional impairment. It is important to appreciate that acute respiratory failure developed at what we consider relatively low airway pressures of 30 cm H_2O, following an apparent improvement (super-normal) within the first 24 hours. Hence, the frequent deterioration in clinical picture seen while on a mechanical ventilator just as likely may be the result of treatment with the mechanical ventilator, and may not represent evolution of the primary underlying disease process.

In collaborative studies in patients conducted at the University of Milano (Dr. L. Gattinoni), it was possible to immediately wean patients dependent on the mechanical ventilator for as long as one month at high airway pressures, to spontaneous breathing with the use of an extracorporeal

membrane lung. These results are a direct extension of our own animal laboratory work, and confirms the overall validity of induced lung disease as a overriding factor in the delay of healing, or the lack of healing, or in the progression to a new disease process. We believe failure of the ECO study was, unfortunately, directly related to lack of this knowledge.

By extension, our results to date raise serious questions about current clinical use of the mechanical pulmonary ventilator, in all age groups. Our results and conclusions need to be confirmed and extended by other investigators, to arrive at standards of management, and for the safe use of this device.

We have continued to improve on the single catheter perfusion system for both animal, and clinical use. This system requires but one vessel (venous) cannulation, and can control breathing so as to dispense with the use of the mechanical pulmonary ventilator altogether. Patients still need to be on CPAP (continuous positive pressure airway pressure) breathing spontaneously, and need no longer be intubated.

Through this multidisciplinary effort combining medicine, neonatology, physiology, pathophysioloy, biomedical engineering, and by designing a specific pulmonary assist device and system, we are changing present understanding of established concepts in the prevention, treatment, and cure of acute respiratory failure.

In collaboration with Dr. Ito, this Section has developed an integrated monolithic countercurrent chromatography system (MICCC) based on differential solubility of compounds in a two phase solvent system. The system is compact, and is likely to assume usefulness in the clean separation of chemical compounds, and biological matter, not attainable by other methods.

Biophysical Instrumentation

This Section has continued its work on developing new, highly precise instrumentation to provide the needed thermodynamic and kinetic data for those scientists attempting to explain the process of metal ion chelation, an important control mechanism in many biochemical reactions that take place in the cell and at the cell wall. When complete, this work should significantly assist those who design therapies for the treatment of diseases in which this control mechanism has been disrupted. The completion this year of the differential thermal-ph automated titration apparatus has permitted us to study the binding constants and heats of reaction of calcium with several important chelators used in many biochemical reaction studies, particularly muscle action and various transport processes in ATPase and Sacroplasmic reticulum. Detailed thermodynamic and kinetic models have been built and partially substantiated for the reaction of calcium and EGTA. EGTA is an organic buffer widely used in the above experiments. However, this latter molecule is proving to be much more interesting from a mechanistic standpoint providing information about the control mechanism itself. Because of this, C^{13} NMR together with X-ray crystal structure determinations are being carried out with the Laboratory of Chemistry, NHLBI. These studies are

presently being extended to the very important biological protein calmodulin which is calcium dependent for its control functions.

Extensive development work has been carried out during the last year on both the batch and flow microcalorimeters in an attempt to define all sources of instrument artifacts. This has resulted in the reduction of these artifacts to the level of about 50 microjoules for the batch, this being due to surface reactions, and less than 10 microjoules for the flow. The surface reactions occur between the Kel-F surface and the various chemicals and proteins which must be used in the determination of various heats of reaction. Tantalum coating of the inside of the Kel-F cell has greatly reduced these artifacts.

The flow microcalorimeter has been improved in the last year through the introduction of a new polypropylene flow cell developed with the help of our section on Pulmonary and Cardiac Assist Devices. A number of improvements were made in the molding process thus greatly reducing the flow induced heating. By taking advantage of new technologies developed by a local company, to protect computer disks from moisture, we have been able to coat the surface of the polypropylene with carbon laid down as a diamond lattice about 2000 Angstroms thick. This has stopped the flow of water through the wall and stopped cooling due to evaporation. Experiments are presently under way with this instrument to provide information on the extremely high specificity of the reaction of t-RNA transferases with their appropriate polyamines. This instrument permits experiments to be carried out on 10^{-9} moles of enzyme present in 50 microliters of solution. Thirty microjoules of heat can be measured with a ten to one signal to noise ratio. Extensive utilization of this instrument is planned for direct binding studies of various proteins and nucleic acids.

Work has continued this year on the development of the inertial drive quench flow and thermal stopped flow instruments. These instruments will be used for the study of calcium reactions in Sacroplasmic reticulum, ATPase, and calmodulin. Cryo-quenching of enzyme reactions will also be carried out. Both the thermal and quench method are useful when there is no optical, fluorescence, or hydrogen ion change in a chemical reaction. Both instruments require only 0.25 ml of reagent per experiment. They both have a time resolution of 1.5 milliseconds at present. It is hoped that the quench system can be reduced to below 0.5 milliseconds in the coming year.

Cell Measurements System

The porous bottom culture dishes (PBCDs) and the devices for the sterile measurement of the electrophysiological parameter of cell layers which we developed are receiving wide application. In addition to the studies of kidney epithelial here at NIH and in more than 50 other laboratories in the U.S.A. and abroad, they are being successfully used in the study of pulmonary epithelia. We have shown that the electrophysiological responses A6 (Xenopus laevis kidney) cell layers are enhanced by increasing the exchange of nutrients and wastes at both the upper and lower surfaces by gently shaking the PBCD's in the incubator during the entire development of the cell layer and its Na transport capability.

The major role played by Ca^{++} in the regulation of transport and other process in cells has prompted us to improve the methods of measuring Ca^{++} activity inside epithelial cells of many kinds. Microelectrodes need to be 0.1 or 0.2 microns in diameter to enter most epithelial cells without causing damage which makes reliable readings impossible. It is logically argued that electrodes of this size have walls so thin that they are totally hydrated near the tip and with the monovalent ions of the glass become Na^+ and k^+ electrodes. This conflict between Ca^{++}, Na^+ and K^+ sensitivities is believed to cause the poor performance often seen for electrodes smaller than 1 micron in diameter at Ca^{++} activities below 10^{-6} molal. We have therefore designed, constructed, and used unique anular burners for making micropipettes form fused quartz. These fused quartz micropipettes are made from high purity material so that no metal ions will be present in the wall to give the completed microelectrodes unwanted ionic responses. It is expected that this will significantly improve the accuracy of intracellular Ca^{++} activity measurements of epithelial cells.

Separation Science Instrumentation

Development of countercurrent chromatography (CCC) has been continued in special emphasis on high-speed CCC which has been recently introduced through our laboratory. Investigations were focused on hydrodynamic behavior of two immiscible solvent phases in a coiled column subjected to a particular mode of synchronous planetary motion. Mathematical analysis showed that the system produces a highly complex heterogeneous centrifugal force field which is determined by a single parameter $\beta = r/R$, the ratio of the coil radius, r, to the orbital radius, R, of the coil holder.

With several different models of the horizontal flow-through coil planet centrifuge devices and a set of conventional two-phase solvent systems, a series of experiments were performed to study effects of orientation and configuration of the column, revolutional speed and radius, various physical properties of the solvent system, etc. under continuous elution of the mobile phase.

The results clearly indicated that the hydrodynamic distribution of the solvent phases is closely related to the hydrophobicity of the nonaqueous phase. In the hydrophobic solvent system such as hexane/water, the upper phase is distributed toward the head and the lower phase toward the tail. In the hydrophilic solvent system such as sec.-butanol/water, the above hydrodynamic trend is reversed. The intermediate solvent systems with moderate hydrophobicity exhibit a variable hydrodynamic trend which is sensitively affected by parameter β. With a small β of 0.125 the lower phase is distributed toward the head as in the hydrophilic solvent system whereas in a large β of 0.75 the upper phase is distributed toward the head as in the hydrophobic solvent system. Studies on the spiral form column indicated that the above head-tail relationships exert major influence on hydrodynamic distribution of the solvent phases, although the effects of the force gradient created by the spiral pitch is by no means negligible.

The above hydrodynamic trend of the solvent system in the centrifugal field was found to have close correlation to the settling times of the solvent

system in the unit gravitational field, thus providing a reliable numerical expression for statistical analysis. The studies showed that viscosity of the solvent system is strongly correlated with the settling times (r= + 0.88) whereas both interfacial tension and density difference between the two phases also gave some degree of negative correlations. Since the viscosity of the solvents is sensitively affected by temperature, effects of the solvent temperature on the settling time were investigated in viscous hydrophilic solvent systems. The results showed that at 50°C settling times of both n-butanol and sec.-butanol solvent systems were shortened within the range for the intermediate solvent systems unless the phase composition approaches near the plait point. This finding promises great improvements in retention of the stationary phase, partition efficiency and sample-loading capacity by performing high-speed CCC under elevated temperatures. The high-speed CCC centrifuge equipped with a temperature control system has been constructed and tested with excellent results.

Stroboscopic observation on hydrodynamic motion of colored solvent phases was performed with both spiral and multilayer coil columns under continuous elution of the mobile phase. At 800 rpm the column was divided into a mixing zone observed in one quarter of each helical turn near the center of the centrifuge and the settling zone in the remainder of the column. The results indicate that the mixing zone continuously moves toward the head of the coil at the rate of column revolution and the solutes present in the column are subjected to a repetitive mixing and settling process at an enormously high rate of over 13 times a second, thus explaining the high partition efficiency produced by the high-speed CCC system.

Efforts to scale up the preparative capacity of the high-speed CCC system have been continued by using a multilayer coil prepared from a larger diameter of tubing measuring 0.55 cm i.d. Using a short column the best operational conditions were determined with a standard set of DNP amino acid samples and a two-phase solvent system composed of chloroform, acetic acid, and 0.1N HCl at a 2:2:1 volume ratio. Two different ranges of β were found to produce satisfactory retention of the stationary phase. With a small β around 0.25, the good retention was produced by eluting either the upper phase in the head to tail mode or the lower phase in the tail to head mode. With large β near 0.5 or greater, good retention was obtained by eluting the column with the lower phase in the head to tail mode or the upper phase in the tail to head mode. Under these operational conditions large-scale preparative separations were successfully performed with a long multilayer coil. The present system may be further scaled up by the use of a larger bore column.

Electron Spin Resonance Development for Medical and Biological Problems

The overall goal of this project is to develop and adapt Electron Spin Resonance Spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. Electron Spin Resonance Spectroscopy is a powerful technique for studying metal metabolism and free radical generation. For

paramagnetic metal ions such as Fe(III), Cu(II), and Mn(II), Co(II) information can be obtained about the redox state of the metal, the spin state, the symmetry of metal binding and the identity of binding ligands. For free radicals information can be obtained about their structure, mobility, stability, and quantitation. The Electron Spin Resonance technique provides critical information on these problems which cannot be obtained in any other way. It has been applied to a large number of biological problems at the in vitro biochemical level, however, the great potential of this technique has not been fully realized due to limitations in sensitivity and sample geometry of conventional commercial spectrometers. With conventional commercial spectrometers only samples in 1-3 mm tubes or flat cells can be studied and the sensitivity is limited to 10^{-4} - 10^{-5} M concentrations of metal ions such as Fe(III) or Cu(II) or 10^{-6} M concentrations of free radicals. In order to study in vivo metal metabolism and free radical generation in intact cells and tissues it is first necessary to be able to accommodate the geometries of these biological samples and then the sensitivity must be optimized in order to be able to detect the concentrations of metals and radicals found within cells. Therefore, we are working on approaches to increase the sensitivity and developing spectrometer and cavity designs suitable for a large range of biological problems ranging from mcirosamples, to cultured cells to whole tissues. While developing these techniques we are demonstrating their feasibility and importance by actively applying them to solve important medical and biological problems.

Over the past year an X-band Electron Spin Resonance Spectrometer has been assembled complete with variable temperature capability from 4°C to 40°C. Work was performed evaluating a number of different cavities and resonators including rectangular TE cavities, cylindrical TE and TM cavities and loop gap resonators.

Over the past year this program has led to the solution of two important biomedical problems. 1. A mechanism has been determined which may explain the origin of the adriamycin induced cardiomyopathy, (Zweier, J. Biol. Chem., 1984, in press). This work may also provide important information regarding the tumoricidal mechanism of adriamycin. 2. The distance between two iron binding sites was measured and this provides insight into the mechanisms of iron transport into all cells of the body (Zweier, 1983, J. Biol. Chem. 258).

Luminescence Instrumentation

The field of fluorescence spectroscopy has been advanced by contributions to methodology and to applications of the techniques. In the area of methods, the following specific projects were undertaken:

1. A new assay for serum albumin has been developed based on its enzymic (esterase) activity in hydrolyzing a fluorogenic substrate. This assay is the most sensitive method for albumin (other than RIA), based on comparisons with reported methods. The substrate we used was Naphthol AS acetate, which produces a bright green fluorescence. The assay had a limit

of detection of 14 picomoles of albumin. Precision, expressed as relative standard deviation, was 8% in the nanomole range. The assay was compared with the Bromcresol Green method, used in many clinical laboratories including N.I.H., using serum samples, and the two methods agreed within experimental error.

2. We have been compiling an atlas of fluorescence spectra of dyes covalently attached to ovalbumin. The atlas will contain excitation and emission spectra and fluorescence lifetimes. There is a great need for a compendium of this sort. We limit ourselves to avalbumin conjugates all prepared in the same way and examined at 23°. The spectra are obtained on an Aminco-Bowman spectrofluorometer and are corrected for detector response and lamp output.

3. A new method called "fluorescence difference decay photometry" has been proposed. A paper has been accepted which describes the advantages of systems where one fluorescence decay curve can be subtracted from another. For example, if a sample is partly quenched by some added quencher like iodide, the difference curve of unquenched-minus-quenched samples will give the decay of the quenched species. The examples in the publication are: sequential dansylation of albumin in which the lifetimes of successively added dansyl groups are determined, partial quenching of alcohol dehydrogenase and BSA to see which of the two tryptophans is quenched first, and dynamic quenching of quinine by chloride ion to show the shape of the fluorescence difference curve in the case of collisional quenching. The method will be facilitated by a high-repetition rate laser-based system.

4. Planning and design for a new YAG laser-based lifetime apparatus has been in progress. Digitization and correction of Aminco-Bowman spectra has been accomplished on a Hewlett-Packard computer.

In the area of applied fluorescence spectroscopy, the following projects have been continued.

1. The mechanism of fluorescence quenching of concentrated dyes encapsulated in liposomes has been elucidated. By surveying a large number of dyes, it was found that only those which seemed to dimerize and also have a highly polar nature were found to be suitable in such dye-liposome systems. Aside form 6-carboxyfluorescein, which was the original dye reported by Weinstein et al, sulforhodamine B and several pyrne sulfonates will be useful fluorescent compounds in liposomes. Sulforhodamine B has the advantage of a long wavelength emission and absence of pH-dependence of its emission in the physiological pH range, in contrast to 6CF, whose pK_a is about 7.4. Pyrene trisulfonate gives an excimer-like emission at high concentrations. The compound was identified as an impurity in a commercial sample of another compound by Dr. Henry Fales, who now is synthesizing it for confirmation. With his help, design and synthesis of dyes having the right balance of polarity and tendency to dimerize could result in other dye-liposome systems useful for following liposome targeting into cells and liposome integrity.

2. The esterase activity of albumins has been further studied. Nobody knows why albumins contain this enzymic activity. One approach to this problem is to see what range of substrates will work. We have found that albumins hydrolyze 4-methylumbelliferone esters (acetate, butyrate, palmitate, oleate, etc.), acetates of Naphthol AS and related compounds, and p-nitrophenylanthranilate. During the hydrolysis, an acylated-albumin is formed. The site of acylation of albumin is different from the site acetylated by aspirin. The Naphthol AS compounds also contain an aryl amide linkage which seems to be hydrolyzed. Could the esterase activity actually be an expression of a peptidase activity? For instance, chymotrypsin and trypsin have proteins and peptides as their natural substrates, but are usually assayed with chromogenic esters. Perhaps the enzymic activity of serum albumin is actually to degrade free-floating peptides such as kallikreins. By surveying a number of fluorogenic substrates and determining their rate of hydrolysis by stopped-flow fluorescence methods, we hope to make the structure-activity relations clearer.

3. Collaboration with other groups has continued. Initial fluorescence decay studies have been done with dansyl-calmodulin obtained form Dr. Randy Kincaid. Data on the fluorescence decay of tryptophans of ornithin transcarbamylase of yeast form Dr. Preston Hensley of Georgetown University, also have shown changes upon binding of inhibitor. These studies will be much speeded up by a laser-based high-repetition rate system.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01404-16 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Lung System for Long Term Respiratory Support

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Kolobow	Medical Officer	LTD:NHLBI
	V. Chen	Mech. Engineer	LTD:NHLBI
	P. Prato	Visiting Fellow	LTD:NHLBI
Others:	M. Moretti	Visiting Fellow	LTD:NHLBI
	K. Tsuno	Guest Worker	LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have performed long term studies on mechanical pulmonary ventilation in laboratory animals, exploring the effects of 50 and 30 H_2O peak airway pressure. At a peak airway pressure of 50 cm H_2O there was uniformly a period of substantial improvement in lung function and lung mechanics over the first few hours of mechanical pulmonary ventilation; following this initial improvement there was progressive deterioration in all measured variables, leading to severe acute respiratory failure. In similar studies at peak airway pressure of 30 cm H_2O, there was a lesser though similar course, leading to respiratory failure in all animals so ventilated. We wish to implicate the mechanical pulmonary ventilator at pressures at least as low as 30 cm H_2O and its use-abuse due to lack of previously available information as the major factor responsible for the delay, or the lack of healing, in such diverse disease states (all treated while on the ventilator) as adult respiratory distress syndrome, hyaline membrane disease, bronchopulmonary dysplasia. The judicious use of the "third lung" (membrane artificial lung) can offer a means to avoid resorting to mechanical pulmonary ventilation permitting diseased lungs to fully recover.

1016

Project Description

Objectives:

To develop a perfusion system for the long term pulmonary and cardiopulmonary assistance.

1. The most widely used clinical perfusion systems for open heart surgery are still based on the bubble oxygenator, where blood is first directly exposed to oxygen. Such systems are not suitable for long term use beyond 6-12 hours, and are limited in part by cellular destruction and clotting abnormalities.

However, blood oxygenators where blood is separated from oxygen by a semipermeable membrane, or a microporous membrane, are inherently more safe and have greatly extended the safe limits of prolonged extracorporeal blood oxygenation, particularly through systems using semipermeable membranes. Those systems in particular are capable of and have been shown to function for over one month without becoming device-method limited. Hence, those systems are ideally suited for the treatment of acute and chronic pulmonary insufficiency.

2. To devise a safe extracorporeal perfusion system for extracorporeal removal of carbon dioxide in the control of alveolar ventilation - control of breathing.

Alveolar ventilation is intimately tied to oxygen uptake and the release of metabolically produced carbon dioxide. Both CO_2, and/or oxygen can selectively be provided for through an extracorporeal membrane lung, eliminating the need for the natural lungs for gas exchange.

It is also possible that some fraction of CO_2 and oxygen can be similarly supplied though the extracorporeal (third) lung, while the remainder of gases can be provided for through some still functional parts of the diseased lungs.

More importantly, metabolically produced CO_2 can be removed from blood through an extracorporeal "third lung", at a blood flow but a fraction of what would be needed were one also to provide for total oxygen delivery through extracorporeal means. Limiting one's efforts to CO_2 removal greatly simplifies the technical aspects of extracorporeal bypass.

This concept is directly applicable to any patient with respiratory failure who is about to become a candidate for mechanical pulmonary ventilation. Such a decision might not have been unreasonable if the mechanical ventilator had been shown not to damage the lungs; unfortunately, such is not the case. The extracorporeal "third lung" is fully capable to augment and/or to fully shoulder total CO_2 removal, thereby avoiding any possible

deleterious side effects from the use of a mechanical pulmonary ventilator. The projected patient population includes adult respiratory distress syndrome (ARDS), neurogenic lung disease, hyaline membrane disease (HMD), meconium aspiration syndrome (MAS), bronchopulmonary dysplasia (PBD), the pump lung syndrome following total cardiopulmonary bypass using bubble oxygenator systems, and other states of acute respiratory failure.

The problem faced in chronic respiratory failure are different in nature. No information is available as yet whether the "third lung" if used for hours, days, or weeks, can help reverse obstructive, destructive, or fibrotic changes occurred over many years, or a lifetime. From published studies and our own studies, it appears likely that perpetuation of a disease state and abnormal ventilation can accelerate the disease process as well as effect multiorgan function. The extracorporeal "third lung" may just be the proper means to "rest" the lungs, and to provide for some degree of reversal of the disease process.

3. Acute respiratory failure: adult respiratory distress syndrome (ARDS): high pressure mechanical pulmonary ventilation.

a. Adult respiratory distress syndrome (ARDS)

This syndrome in its severe form carries a high mortality, with little or no progress in improving on its severe mortality over the past 10 years. In a multicenter controlled NHLBI funded study, patients meeting criteria of severe ARDS had a 90% mortality. Those results were uniform among the many centers involved in the study, and only attests to the complex nature of severe acute respiratory failure. Of more interest is the finding that the use of an extracorporeal "third lung" did not alter the ultimate outcome in a randomly selected population of patients with ARDS, as part of the ECMO study.

The clinical picture of ARDS can be caused by a variety of physical, biological, and chemical factors. The overall results are a non-compliant, stiff lungs, that are most difficult to ventilate. Unfortunately, those lungs must be ventilated for the removal of metabolically produced CO_2. The difficulty arises from increase in physiological dead space, high airway pressures, and what is generally called barotrauma. The latter term refers to a multiplicity of pulmonary parenchymal damage, alteration in CNS control in ventilation, ventilation/perfusion mismatch, cardiovascular, hepatic and renal impairment, to name a few. Thus, the "mere" inclusion of a mechanical pulmonary ventilator not only accomplishes alveolar ventilation as planned, but also opens the road to a multiplicity of pathophysiological aberrations that in the end are the predominating feature of ARDS - fluid retention, oliguria-anuria, hepatic dysfunction with bleeding and clotting abnormalities, and severe blood gas abnormalities.

Such present day clinical use of the mechanical ventilator can be counterproductive. The few laboratory studies published some decades ago have raised sufficient question about safety of those devices, only soon to be forgotten and ignored among the proliferation of newer and "better" models of mechanical pulmonary ventilators.

Acute lung failure in adult, child, and infant need to be managed to achieve full recovery as the eventual goal. Such a goal does not include treatment based only on a few arterial blood gas measurements: improvement in arterial blood gases can be obtained in almost every case by altering ventilator settings. Unfortunately, such improvement is most commonly transitory, with further impairment surely to follow. The goal of the treatment of ARDS is the elucidation of the many factors at play that contribute to the delay, and the lack of healing, of the lungs while on a mechanical pulmonary ventilator. No such difficulty exists when the mechanical ventilator is dispensed with altogether though the use of an extracorporeal "third lung", eliminating delay, or lack of healing, in patients on mechanical pulmonary ventilator.

b. High pressure ventilation with a mechanical ventilator as the cause of lung disease.

In any type of early acute lung failure it is common to find areas of parenchyma that is little or not affected by the disease process. And yet, during mechanical pulmonary ventilation the entire lungs, diseased as well as the remaining healthy area of the lungs are exposed to mechanical pulmonary ventilation at potentially high airway pressures. Such exposure can rapidly lead to acute lung failure, with a poor outcome.

Such a scenario need not develop if lungs are never to be exposed to a mechanical pulmonary ventilator, so that the patient can continue to breathe on his own, and while sufficient amount of CO_2 is continuously removed by an extracorporeal "third lung".

In our previous studies in sheep we have shown the rapid loss of pulmonary function, leading to death well before 48 hours, and when sheep were ventilated to a peak airway pressure of 50 cm H_2O. It is self evident that ventilator lung disease cannot exist when no ventilator is used at all.

Methods employed and major findings.

1. Blood access has been of major importance in any extracorporeal perfusion system. In the conventional system, access occurs through a vein and an artery, or through two veins. We have developed the single catheter venous access system that requires one venous cutdown, and yet that allows sufficient extracorporeal blood flow to maintain arterial blood PCO_2 in the normal range. Blood flow in this system is controlled through occluding

clamps. The system as designed and fabricated in this laboratory has proven to be safe, reliable, and suitable for extracorporeal CO_2 removal to be particularly suitable in newborn infants, where it becomes not possible to spare more than one vein without significant untoward effects.

2. Adult respiratory distress syndrome (ARDS)

These studies have been carried out in collaboration with former Visiting Fellows from Italy, now at the Institute of Anesthesia and Intensive Care in Milano, Italy.

The patient population is almost exclusively a referral population, all meeting or exceeding ECMO entry criteria. As reported previously in some 40 patients treated by this group, some 70% have been long term survivors (expected survival: 10%).

Following our finding of likely iatrogenic lung damage from mechanical pulmonary ventilators from this laboratory, they have now accepted also patients with severely diseased lungs who could not be weaned off the mechanical pulmonary ventilator for over a month. On placing those patients on the extracorporeal "third lung" for partial CO_2 removal, these patients were weaned immediately, and bypass could be discontinued after some 3 days.

Those clinical studies implicate conventional mechanical pulmonary ventilators in the delay, and the lack of healing, in a significantly large patient population. The Additional Data Collection (ADC) of the ECMO study referred to above explored the ultimate outcome in patients with substantially milder forms of acute lung failure (not meeting ECMO criteria): by the end of one month, some two thirds of that patient population of some 700 patients had died. We believe that the great majority of the ADC patients to have been prime candidates for partial CO_2 removal, with an excellent chance of survival.

3. Lung injury from moderate pressure mechanical pulmonary ventilation.

We have exposed paralyzed, sedated sheep to mechanical pulmonary ventilation at peak airway pressures of 30 cm H_2O. This choice was made assuming this level of peak airways pressure to be harmless, and because those airway pressures are frequently found in most hospital settings, without undue concern from iatrogenic lung disease.

We have explored our findings in 5 groups of animals. Group A - peak airway pressure kept at 30 cm H_2O and respiratory rate adjusted to 4-7 min to keep arterial blood PCO_2 in the normal range. Group B - peak airway pressure 30 cm H_2O, with positive end expiratory pressure (PEEP) of 10 cm H_2O, and a rate adjusted as needed; group C - as in group B except PEEP of 20 cm H_2O; group D - as in group A but ventilated with added 3.8% CO_2;

group E - ventilated at a respiratory rate of 15/min with a variable dead space to keep arterial blood PCO_2 in the normal range, at airway pressure of 30 cm H_2O. Group E - control group, ventilated at normal respiratory rates and tidal volumes, and normal airways pressures of some 15 cm H_2O. The control group of sheep faired excellent, and showed normal lung function and gross and microscopic findings at elective autopsy some 48 hours later.

All the study group animals, group A to D, as a group had severely impaired impaired lung function, severely depressed functional residual capacity (FRC), very low lung compliance, marked pulmonary atelectasis, hypercapnea, and hypoxia. These changes almost always were preceded by an apparent improvement in all measured variables, during the first 24 hours of mechanical pulmonary ventilation, only to progressively to deteriorate, with chest X-ray changes diagnostic of ARDS. Of interest is the minimal, or no sparing found in group B and C animals with added PEEP. While blood oxygenation may have been benefited by PEEP, this did not prevent, or delay the relentless progression of ARDS.

As a side issue, the model of lung injury from mechanical pulmonary ventilation provides us with an excellent means of creating and thus assessing the benefit of other means of treating damaged lungs. It is doubtful, that damage so caused by mechanical ventilators can be reversed by any future technical improvement in the design of a mechanical ventilator. Alternatives, such as high frequency pulmonary ventilation, and the extracorporeal "third lung" are the likely means to play a role in reversing damage induced by mechanical pulmonary ventilators.

Significance to Biomedical Research and the Program of the Institute:

This laboratory has previously developed the technology for the design, and construction of safe perfusion systems, which systems include cannulas, tubes, blood surfaces, membrane artificial lungs (the "third lung"), and has demonstrated their safety in long term animal studies. This technology cannot be transferred to the bedside without an appreciation of the pathophysiology of acute lung disease. The best devices in the world, the best perfusion team, and the best of everything will be to no avail if an offending agent (the mechanical pulmonary ventilator) is not so identified, and removed.

Our studies have systematically implicated a device (mechanical ventilator), and in appropriate spontaneous hyperinflation (see last year's report) as an as yet unrecognized offending agent.

In our last few annual reports, we have also systematically explored possible mechanisms of respiratory failure in the preterm fetal lamb population, in meconium aspiratoin sysndrome, and in bronchopulmonary dysplasia. We have shown that "conditioning" of the lungs through

prolonged pulmonary insufflation to be a key variable at increasing FRC and lung compliance, and in initiating normal pulmonary ventilation; and our great caution in the use of high airway pressures in both newborns, and in MAS, it being the prime culprit in the development of BPD.

Thus, engineering technology to develop new and improved systems and methods must go hand in hand with our enhanced understanding of the deleterious side effects through on by potentially lethal use-overuse of the devices themselves. The perceived cry for "better systems" does not absolve one form exploring factor(s) that in the long run may turn out to be the true holdups.

Our concept in the control of breathing through the sue of an extracorporeal "third lung", the elucidation of mechanisms leading to unstable lungs with large dead space, and intrapulmonary shunting, have greatly enhanced our understanding of the evolution of acute lung disease. Pulmonary failure is the major cause of neonatal morbidity and mortality; our contribution once widely disseminated, is likely to change that significantly. Success in the adult age group patients suggests the emergence of systems specifically tailored for their needs, reducing morbidity and mortality. In a rare sidelight to technological progress, the overall cost of treatment is likely to be significantly reduced.

Proposed Course:

We will continue to enhance our understanding in the pathophysiology cause, prevention, and treatment of acute respiratory failure using extracorporeal "third lung" perfusion system. Studies are to be initiated to apply similar concepts to the treatment of acute partial, or total myocardial failure.

Publications:

1. Solca, M., Kolobow, T., Huang, H. H., Chen, V., Buckhold, D., and Pierce, J. E.: Respiratory Distress Syndrome in Immature Lambs: Prevention through Antenatal Accelerated Conditioning of the Lung. Am. Rev. Resp. Diseases, 129: 979-981, 1984.

2. Kolobw, T., Moretti, M., Mascheroni, D., Chen, V., Buckhold, D., Pierce, J.E., Fumagalli, R., and Ting, P.: Experimental Meconium Aspiration Syndrome in the Preterm Fetal Lamb: Successful Treatment using the Extracorporeal Artificial Lung. Trans. Am. Soc. Artif. Internal Organs, 29: 221-225, 1984.

3. Solca, M., Kolobow, T., Huang, H., Pesenti, A., Buckhold, D., and Pierce, J. E.: Management of Antenatal Preterm Fetal Lung in the Prevention of Respiratory Distress Syndrome in Lambs. Biology of the Neonate, 44: 93-101, 1983.

4. M. Joris, Vanderhoeft, P., Vanstratum, M., Plasman, C. H., and Kolobow, T.: Development and Analysis of a "Push-Pull" Venous Extracorporeal Membrane Assistance of Respiration (VECMAR). European Soc. for Art. Organs, in press.

5. Kolobow, T.: ECMO - Does it Save Lives? Proceedings, International Society for Artificial Organs, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01407-21 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less Title must fit on one line between the borders)*

Luminescence Spectroscopy in Biomedical Research

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	R. F. Chen	Senior Investigator	LTD:NHLBI
Others:	H. Fales	Chief, Lab. of Chemistry	CH:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Fluorescence quenching of dyes encapsulated in liposomes has been studied to find dyes which will have different spectral properties inside and outside the liposomes. Several dyes have been identified and the common characteristic is that they dimerize at high concentration, causing a change in fluorescence. Two pyrene sulfonates have been found causing a change in fluorescence. Two pyrene sulfonates have been found which have excimer-like emission at high concentration. Spectra and lifetimes have been measured for all dye-liposome systems.

The esterase activity of human and bovine serum albumins has been studied with fluorogenic aryl esters. These esters include those of 4-methylumbelliferone, p-nitrophenylanthranilate, and Naphthol AS and its derivatives. Rate constants for the hydrolysis by BSA and HSA have been recorded in order to correlate substrate structure with enzymic activity. Acyl-albumin intermediates are formed with each substrate at the same site on albumin. The site is distinct from that acetylated by aspirin.

Fluorescence lifetime and anisotropy studies have been initiated on the proteins ornithine transcarbamylase of yeast, and brain calmodulin. Information has been obtained on interaction of the proteins with inhibitors or other proteins by the change in properties of intrinsic fluorescence of armomatic amino acid residues or extrinsic emission of covalent labels such as DNS.

1024

Project Description:

Objectives:

The purpose of the work is to study specific problems of importance in biomedical research, using sophisticated techniques of luminescence spectroscopy. In addition to advancing knowledge in specific areas of biochemistry, this work furthers development of instrumental methods.

Methods Employed:

Fluorescence and absorption spectra were obtained with Aminco-Bowman and Cary spectrometers. Lifetime data were obtained with modified Ortec single-photon counting lifetime apparatus. Data reduction was performed with laboratory computers (SOL and DEC MINC) and through the DEC 10 system of the computer center. All chemicals were obtained commercially; no human or animal experimentation was involved.

Major Findings:

1. Work has continued on the characterization of dye-liposome systems. In 1977 Weinstein et al described the properties of 6-carboxyfluorescein (6CF): when incorporated at a concentration of 0.2 M in liposomes, the dye is 97% quenched. Upon release from the liposomes, 6CF fluorescence intensity returns to normal. Since their original report, 6CF-liposome system has been widely used, but no other dyes have been described to substitute for 6CF, nor is it clear why 6CF has the requisite properties. We tried many dyes and have concluded that the concentration quenching is due to dimerization at high concentrations. Sulforhodamine has similar properties, possesses an orange fluorescence, and may be useful in double labeling experiments. Two other compounds were also found to form useful systems with liposomes, namely, the tri- and tetrasulfonic acids of pyrene. The latter dyes seem to dimerize at high concentrations so that liposomes containing them exhibit an excimer-like green emission. Upon lysis of the liposomes, the green emission disappears in favor of the blue monomer fluorescence. The fluorescence lifetimes of the excimer emissions are 23 and 46 nsec, compared with 15.5 and 12.7 nsec for the monomers, for the tri- and tetra-sulfonates, respectively. In each case, a collisional mechanism for the concentration quenching has been ruled out by lifetime data. The pyrene trisulfonic acid was accidentally found, since it is not available commercially. It was found in a bottle labeled "8-hydroxypyrene-1,3,6-trisulfonic acid" and identified by Dr. Henry Fales by Californium induced mass spectrometry, IR, thin layer chromatography, and mainly NMR. He is presently synthesizing the compound, which should be very useful in the liposome system.

2. Work has continued on the hydrolysis of fluorogenic esters by serum albumins. Serum albumins have a little-known esterase activity, which has been described mainly for compounds related to p-nitrophenyl acetate (PNA). We previously found a number of fluorogenic esters which could serve as substrates. The importance of this esteractic activity may have been underestimated: The activity could be of importance physiologically and clinically, and from an evolutionary viewpoint, albumin could be the progenitor of some enzymes having hydrolytic activity. The fluorogenic compounds we have used include p-nitrophyenylanthranilate, 4-methylumbelliferone esters, and esters of the Naphthol AS series. Aside from recording the fluorescence spectra and lifetimes, we have studied the kinetics of the reactions to learn about structure-activity relationships. In each case, reaction with albumin is biphasic, with the rapid formation of an acyl-albumin complex followed by a slower hydrolytic phase. The first order rate constants reported by Means and Bender for p-nitrophenylacetate are 2×10^{-2} and 5.4×10^{-4} sec^{-1}, while we obtain for 4-methylumbelliferone acetate (4MUA) 1.4×10^{-3} and 2.3×10^{-4} sec^{-1}. The Michaelis constants were also measured and suggest that 4MUA is more strongly bound than PNA. Rate constants have also been determiend for the other fluorogenic substrates. Competition experiments have shown that the acylation occurs at the same site on albumin. In contrast, aspirin, also an aryl ester, has been reported to acetylate albumin, but does so at a different site. These studies further our knowledge of albumin and enzymatic action and at the same time utilize fluorescence spectroscopy, decay time measurements, and stopped-flow kinetics.

3. Fluorescence lifetime measurements have been made on two proteins which have been supplied by other laboratories:

1. Ornithine transcarbamylase (OTC) of yeast, which has been purified to homogeneity in large quantity by Dr. Preston Hensley of Georgetown University, and 2. Calmodulin, obtained by Dr. Randall Kincaid from bovine brain. OTC has 2 tryptophans and the fluorescence is modulated by the binding of substrates and inhibitors, suggesting conformational changes. Calmodulin has no tryptophans, but can be labeled specifically by fluorescent dyes such as DNS. In both cases, the fluorescence decay curves as well as anisotropy decay curves can potentially give information about flexibility and conformational changes occurring at specific regions of the proteins. When OTC native fluorescence is quenched by binding of an inhibitor, the lifetime is decrease in proportion to the quantum yield, thus ruling out a static quenching of a subpopulation of the molecules. DNS-calmodulin was supplied by Dr. Kincaid with a degree of labeling less than 1.0. Nevertheless, the fluorescence decay curve was resolvable into two lifetimes, in agreement with the finding that two sites are labeled. These two sites probably are affected in a different way upon binding of calcium ion and calmodulin-dependent enzymes.

Proposed Course

The liposome-dye and esterase projects will be brought to completion. The collaborative projects on ornithane transcarbamylase and calmodulin will be much facilitated by the development of the laser-based fluorescence decay system. Application of difference decay and time-resolved anisotropy techniques will yield much interesting information.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01408-19 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Methods in Fluorescence Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. F. Chen	Senior Investigator	LTD:NHLBI
	C. H. Scott	Biologist	LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new assay for serum albumin has been developed based on its enzymatic activity in hydrolyzing a fluorogenic naphthol substrate. The method correlated well with the Bromcresol Green method which is usually used in clinical laboratories. However, the new enzymic fluorescence method is more sensitive, with a limit of detection of 14 picomoles of albumin.

A spectra and lifetime atlas is nearly complete for a number of covalently attached fluorescent dyes each as DNS, o-phthalaldehyde, fluorescamine, fluorescein, rhodamine B, eosin, etc. Data have been accumulated for ovalbumin labeled under standard conditions and examined at 23°. The spectra were obtained on Aminco-Bowman spectrofluorometers and corrected for instrumental non-linearities.

A new method, "fluorescence difference decay" photometry, has been developed. The method is analogous to difference spectroscopy in absorption or fluorescence. When a sample is partly quenched, the decay curve of the quenched sample is subtracted from that of the unquenched sample to give the decay curve for the fluorophor which was quenched. Variations of the method are useful to resolve complex fluorescence decays, which characterize most biological samples.

Planning and design for a new YAG-laser based single-photon lifetime apparatus has been in progress. Computer support has been updated. The system components will have arrived before the end of the period of this report. Advantages of the system are high repetition rate (megaHz), high intensity, narrow pulse duration, reliability, and pseudo double beam operation.

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Project Description:

Objectives:

The aim of this project is to develop methods of fluorescence assay which will be useful in biomedical research. Apart from establishment of new techniques, the project will also encompass the evaluation of reagents, the analysis of precision and error, and the proposal and characterization of standards.

Methods Employed:

Fluorescence measurements were made with various modified Aminco-Bowman spectrofluorometers, and the Ortec single photon nanosecond fluorometer. Data were reduced with dedicated or time sharing computers. Chemicals were obtained commercially and did not involve human or animal experimentation.

Major Findings:

1. We had previously described methods for calibration of Aminco-Bowman spectrofluorometers in order to obtain corrected excitation and emission spectra. Because of the large number of fluorescent dyes now available it seemed appropriate to construct a spectral atlas which could serve as a reference, if the spectra were corrected. The fluorescence characteristics depend on which protein the dye is attached to, the degree of labeling, the solution conditions, and other factors. Therefore, we have limited our spectra to those involving ovalbumin, labeled under standard conditions, and examined at room temperature at pH 7.4. Data have been accumulated on excitation and emission spectra, degree of labeling, and fluorescence lifetimes of a number of conjugates, including those of the following fluorescent labeling compounds: dansyl aziridine, dansyl chloride, eosin iodoacetamide, fluorescein isothiocyanate, rhodamine B isothiocyanate, thiolyte MQ and MB, Fluram, IAEDNS, coumarinylphenyl maleimide, pyrene derivatives, orthophthalaldehyde, Lucifer Yellow, etc. When finally compiled, the atlas should be a popular reference source for lifetime and spectral data.

2. A novel fluorometric assay has been developed for serum albumin, based on its esterase activity. Serum albumin has a little known esterase activity against aryl esters such as p-nitrophenylacetate. We found that human and bovine albumins hydrolyze Naphthol AS acetate, resulting in a fluorescence excited at 320 nm and monitored at 500 nm. The assay is conducted at pH 8.0 in the presence of cetyltrimethylammonium bromide, a detergent which activates the reaction. Other esterases in serum require either calcium or a higher pH for activity. The assay is conducted with albumin diluted to about 10^{-7} M or less, thus dissociating potentially interfering ligands including bilirubin and fatty acids. The method gives results with serum which correlate well with the widely used Bromcresol

Green method. Relative standard deviation was 8%. Limit of detection for HSA was 14 picomoles, which gave a signal-to-noise of 5.

3. The fluorescence decay curves of most biological materials are complex, reflecting the fact that emission occurs from more than one fluorophor. The problem of how to separate out each component was approached by the use of difference decay curves. The time-correlated single photon counting method we normally use to obtain decay curves permits us to subtract a "reference" curve from a "sample" curve to yield a difference decay curve. A model 3-component system (6-carboxyfluorescein, pyranine, and DNS) had a complex fluorescence decay curve from which the individual decays were obtained by subtracting the decay method to be valid, the following requirements had to be met: use of the same instrumental settings for all solutions, low counting rate to avoid pile-up error, absence of inner filter effects, and lack of chemical and physical interaction such as energy transfer. The difference decay method was shown to be useful for the following systems: 1. Sequential dansylation of serum albumin, in which the lifetimes of the individual groups could be determined. 2. Pure dynamic quenching, where the difference decay curve reveals the lifetimes of both the quenched and unquenched species. 3. Quenching of the fluorescence of liver alcohol dehydrogenase and bovine serum albumin by acrylamide. Both proteins contain 2 tryptophans, one of which is preferentially quenched. The difference decay curves reveal the lifetime of the quenched species. In LADH, tryptophan 314, an exposed residue with a lifetime of 7.2 nsec matches the characteristics of the acrylamide quenched residue. The fluorescence difference decay method in theory has the advantages of other difference spectral techniques for resolving complex signals. This method will be greatly facilitated by our planned laser-based high repetition rate decay time apparatus, to be assembled by Dr. Knutson. Present instrumentation is limiting due to the single-beam nature of the system; the high rep rate of the laser system permits rapid switching between samples to give pseudo double-beam operation.

4. YAG laser pumped dye laser single photon fluorescence decay system. Planning and ordering components for sophisticated fluorescence decay system has been in progress with Dr. Jay Knutson. This system has the advantages of high repetition rate (megaHZ), high intensity, short pulse duration (0.2 nsec), reliability, and automated switching of samples. The supporting computer systems have been reviewed (DEC, MINC, various microcomputers, and the DEC 10 system of DCRT). Dr. Knutson has made it possible to access the extensive fluorescence software at Johns Hopkins University, much of which he wrote. It is expected that when the system is functional, it will be the premier instrument of its kind.

Significance to Biomedical Research and the Program of the Institute:

Fluorescence spectroscopy has played an important role in biomedical research, and the Institute has been in the forefront for many years in developing this technique. The present activity is a continuation of this tradition, which has led to many advances in biology and medicine.

Proposed Course:

The forthcoming year will see the development and testing of the laser-based single photon counting fluorescence decay system (with Dr. Knutson). Specific tests will include extension of our work on difference decays, as well as fluorescence lifetime and anisotropy determinations. With Mrs. Scott we plan to complete the fluorescence spectral atlas of covalent protein labels.

Publications:

1. R. F. Chen: Fluorescence Difference Decay Curves: Resolution of Complex Decays, Anal. Lett., in press.

2. R. F. Chen and C. H. Scott: Fluorimetric Assay for Serum Albumin Based on Its Enzymatic Activity, Anal. Lett., In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01413-22 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Development of Biophysical Methods for Study of Bio-Macromolecular Reactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboretory, and institute affiliation)*

P. I.: R.L. Berger Chief, Biophysical Instrum. Section LTD:NHLBI

COOPERATING UNITS *(if any)* Lab. Molecular Biology (J. Froehlich), Lab. Molecular Carcinogenesis (E. Friedman), Univ. of Maryland (E. Bucci), Univ. of Pennsylvania (L. Thiebault), Old Dominion Univ. (G. Hoy), Biomedical Engin. & Instrum. Branch (H. Casio), and Commonwealth Technology, Alexandria,VA.

LAB/BRANCH

Laboratory of Technical Development

SECTION

Biophysical Instrumentation Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use stenderd unreduced type. Do not exceed the space provided.)*

A new inertial drive flow system has been developed for doing quench flow reaction kinetics in the investigation of the mechanism of reaction of Sacroplasmic Reticulum, ATPase, and other enzyme systems which cannot be followed by optical means. A time resolution of better than 1.5 ms has been achieved to date using the acid quenching of the reaction of 2-4 dinitrophenyl acetate as the test reaction. 0.25 ml of each reagent are required per data point in contrast to 2.5 ml for other instruments. Extension of its use for cryoquenching at -40°C is planned for the coming year. The use of this drive in our zero pressure drop thermal stopped flow system was reported last year. Pressure, velocity, and thermal measurements are underway over a series of viscosities to provide information needed for the support of the ball mixer theory. In addition optical fiber sensors will be added in the coming year so that simultaneous thermal-optical stopped flow experiments may be done.

1032

Project Description:

Objectives:

The major objective of the work of this section is to develop new instruments, analysis methods, and data handling techniques for the physiochemical study of biological reactions. The mechanism of a reaction, i.e. the underlying physical and chemical processes involved, is considered to be understood when a) the structural properties of the reacting molecules are known. In biological systems, a knowledge of how the structure-function relation works to regulate the rate of the reaction is also of vital interest as this is how catalysis is controlled. This section is particularly interested in model reactions because it is believed that considerable progress can be made in developing a more general theory of structure-function regulation if these reactions are understood in detail. The reaction of hemoglobin with various ligands and effector molecules (small molecules which alter the structural properties of the large protein molecule) is used as the principle model of a regulatory protein reaction. Other enzymes are used from time to time if they are available in the same high purity as hemoglobin. The reactions of various cellular enzymes, such as ATPASE, both Na, K transport and Ca transport as well as calcium regulation in calmodulan, are studied as they relate to regulation.

Methods Employed:

Biological physics attempts to find the simplest explanation for complex systems. Toward this end model biological molecules are chosen which, in general, are readily obtained, highly purified, and for which some structural information is available. Systems are chosen to work on for which several possible theoretical models exist to describe their mode of action. Methods are devised to provide the most accurate data possible to assist in deciding between these theories. Since the models chosen are of great biological interest, the instruments and methods generally become useful for the study of other systems as well. This section interacts with other laboratories in order to demonstrate feasibility of these models for their studies and assists commercial companies in producing these instruments for general use. The methods used in the investigation of the mechanisms of enzyme action are primarily those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reaction by optical, thermal, pH electrode, etc. detectors. In general, equipment do to this is not available, either in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory. Construction is carried out wherever most appropriate, i.e., in commercial firms, in other government agency shops, such as NRL or

NBS, or at university shops. In pursuing these investigations, a wide variety of physical parameters must be studied in order to understand the underlying physical theory governing the reactions. Expert consultants are used to assist where needed and biological collaborators join us to insure the proper handling of the biological molecules.

Major Findings:

The need to study reaction paths in macromolecular reactions becomes particularly important when the biological or chemical synthesis of a protein is contemplated. These details often can only be studied using so-called "pre-steady state kinetics". That is simply to say that if enzyme A reacts with substrate B to make C which reacts with a second substrate D to generate E details of A plus B, which is normally a very fast reaction and is thus treated as constant in the presence of large amounts of B and D, must be known. This fast step occurs in microseconds to milliseconds.

The development of a novel new flow apparatus called the "Inertial Drive Stopped or Quench Flow Apparatus" has been undertaken to overcome some of the problems of existing instruments. These problems include poor mixing of viscous solutions, stopping artifacts, and the use of large amounts of material in the case of the Quench Flow System.

The development of this system has proceeded to a point where extensive testing of the unit operating as a quench flow apparatus is now being done. The results to date basically demonstrate that using 0.25 ml of each reagent and quenching with 0.5 ml of 2.5 m HCl a quenching time of 1.5 milliseconds can be achieved. Using two sets of tubing and 3 rotation speeds data points from 1.5 milliseconds to 70 milliseconds may be obtained. The apparatus will next be tested in the Laboratory of Molecular Ageing, NIA, to ascertain its usefulness for Brush border membrane studies and Sacroplasmic Reticulum studies of calcium reactions. Tests designed to explore the apparatus' ability to quench a reaction using a cryoreagent at -40°C will also be carried out.

Work has progressed on the rebuilding of the Interial Drive Stopped-Flow Thermal-Optical System. New observation tubes have been constructed as well as a new drive-stop system. This system will be tested later in the summer at which time thermal, velocity, and pressure measurements will be determined and correlated with a theoretical model of turbulent flow. Our contention is that the mixing of two solutions is primarily achieved by converting the linear momentum of even an already turbulent flow into angular momentum in such a way as to make the radii of the solution domains as small as possible and as close to each other as possible since the

distance is what determines the reaction domain. A major problem will be finding a good method to measure these radii. We believe that with the addition of optical fibers we can devise a means of making this measurement. The optical path for both absorption and fluorescent measurements will greatly augment the thermal detection system, particularly for the study of reactions of calcium chelators since there is often a hydrogen ion released which can be followed by a pH indicator dye.

Significance to Biomedical Research and the Program of the Institute:

Molecular biology and biological physics study the underlying physical and chemical processes involved in biological reactions at the cellular level. This section concentrates on developing new methods that will permit careful measurement of these reactions so that theories of the reaction mechanisms can be critically tested. These theoreis will lead to a far better understanding of how the cell functions and how the fundamental processes of structure and function interact to determine the rate of catalysis which ultimately determine all bodily functions from synthesis to metabolism. As often happens in research, instruments produced with one area in mind turn out to be useful in other areas. One such example is the differential pH apparatus developed in this section which has now been adapted to six clinical tests for certain enzymes and substrates. The instruments to do this are manufactured in Europe and already in use in 30 hospitals.

Proposed Course:

The time resolution of the quench flow apparatus will hopefully be pushed to its design value of 0.5 ms. Optical fibers will be installed in the thermal stopped flow and work started using this instrument on the calcium-calmodulin reactions.

Publications:

Balko. B., Bucci, E., Berger, R. L., Swarzendruber, L.J., and Montemarano, J.X.: J. Biochem. Biophys. Methods, in press, 1984.

Smith, P.D., Liesegang, G. W., Berger, R. L., Czerlinski, G., and Podolsky, R. J. Anal. Biochem., in press, 1984.

Berger, R. L., Clem, T., Harden, V., and Mangum, W.: Methods in Biochemical Analysis, Vol. 30, 269-331, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 01414-12 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Biocalorimeters for Solution and Cell Biochemical Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	R.L. Berger	Chief, Biophysical Instrum. Section	LTD:NHLBI
	T. Kolobow	Medical Officer	LTD:NHLBI

COOPERATING UNITS *(if any)*

Biomedical Engin. & Instrumentation Branch, DRS, (C. Mudd), Northwestern Univ. (M. Marini), Penn State Univ. (N. Davids), N. M. Univ. School of Medicine (P. Simons), and Commonwealth Scientific, Alexandria, VA.

LAB/BRANCH

Laboratory of Technical Development

SECTION

Biophysical Instrumentation Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.55	.55	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The development of flow and batch calorimeters has continued during the past year to the point that mixing artifacts in the 2 ml batch cell have been reduced to plus or minus 40 microjoules. Modification of this unit to serve as a metabolic calorimeter for the study of cell reactions will be started in the coming year. Optical pH, PCO_2, and pO_2 probes will be installed so that a constant environment may be maintained while at the same time measuring the consumption or production of these substances by the metabolizing cells. This can then be correlated with the heat produced to determine the thermochemistry of various substrate reaction paths. We hope then to be able to study the inhibition of these paths by various drugs.

The Flow microcalorimeter has been improved considerably in the last year. A new molded polypropylene flow cell has been developed and tested. Flow artifacts are less than 10 microjoules in the differential mode. 30 microjoule electrical signals can be detected with a signal to noise ratio of ten to one. Diamond coating of the polypropylene flow cell has stopped water evaporation. This instrument will be used for the study of protein reactions both with small molecule substrates and with nucleic acids. In particular, the study of the specificity of polyamine t-RNA transferase is underway.

1036

Project Description

The objectives of this project are to develop methods of measuring the enthalpy of both solution and cell reactions. The many types of reactions which occur even in the simplest system must be identified so that the heat measured is, in fact, that produced by, say a binding reaction, and not the binding plus the heat of ionization of a titratable group which is titrated because a hydrogen ion is released by the reaction. This project is also aimed at utilizing the calorimeter as a modern Warburg respirometer with a thousand fold increase in sensitivity. This would allow detailed metabolic studies to be made including the effects of drugs on the reactions once the thermochemistry of these reactions is known. Thus, initial screening of pharaceuticals could be done using liver, kidney, and other cells.

Methods Employed:

Utilizing various biochemical and cellular reactions, as model system, appropriate instrumentation is designed and built in this laboratory where close interaction between investigator and instrument developer can be carried out. A close working relationship is maintained between this section and BEIB in the design and fabrication of new detectors, amplifiers, etc. At the same time wide use is made of commercial, academic, and other government agencies laboratories both to take advantage of the latest new technology and also to obtain, when possible, a significant savings for the government. This section specializes in being aware of the latest physical techniques being developed for other work and then adopting those methods, where appropriate, for biological studies. In addition to instrument development, considerable effort is put into data collection and analysis. Thus microprocessors are used when they will significantly improve the quality of the data taken. We have developed a simulation technique for analyzing very complex biological problems, particularly those involving heat conduction, or diffusion, and simultaneous chemical reactions. This is called the D-B Finite Element Simulation Technique or FEST. This method is employed in all of the systems developed.

Major Findings:

The study of the reactions of proteins with various ligands and effectors molecules is necessarily dependent both upon advances made in protein purification and upon instruments to observe the reactions. Several important questions have gone essentially unanswered over the years more from a lack of instrumentation and of an understanding of the physical chemistry of proteins in solution than from the purity of the protein. One of these concerns the role that water plays in macro-molecular reactions. Water is gradually being recognized as a major constituent of the energy.

system which affects the interaction between all kinds of materials in solution. A good example is the interaction potential between DNA molecules as presently being elucidated by Parsegian, (DCRT). Basically, the question arises when a protein or nucleic acid undergoes a conformational change upon reaction and the energy to drive this change does not appear to be available in the reaction bond energy. Two possible examples of this are the Ca^{-}Calmodulin reaction with various proteins and the reaction of oxygen and hemoglobin. To address this problem we have been attempting to construct microcalorimeters which would on the one hand give the total heat of the reaction and on the other hand follow the thermokinetics of the reaction. Such a measurement is essential if the heat of reaction is to be determined because the more general method of ascertaining heats of reaction - Van't Hoff plots - are not reliable since the Van't Hoff's can be made over such a small temperature range with a protein. If structural changes occur the heat capacity of the system has changed and thus ΔH from Van't Hoff plots are meaningless. Whey should there be an interest in the heats of reaction? The equation which governs all reactions in solutions and solids is that the Gibbs free energy, ΔG, that is, will the reaction "go" spontaneously and thus produce energy or does energy have to be supplied, is determined by knowing the equilibrium constant of the reaction.

$$\Delta G = -RTLnK$$

Under conditions of constant pressure ΔG is also equal to the change in the heat of reaction minus the change in entropy times the temperature, i.e.

$$\Delta G = \Delta H - T\Delta S$$

ΔS relates to the conformational changes that occur during the reaction. In the past this reaction scheme has been expressed ignoring water. The effects of salts may be included, but are generally ignored. Thus if K and ΔH can be measured and the effects of water and salts sorted out the conformational change as given by ΔS can be determined. Eventually this can be correlated by x-ray, replacement or other methods of structure determination. Knowledge of these structural changes are particularly important when synthesis of a protein is attempted, or when drugs are being designed to interact with a protein to block enzyme activity such as in cholesterol synthesis, etc. This is also important in a multifunctional enzyme such as calmodulin when its interaction to control Ca activity in cardiac muscle may be entirely different from its control of Ca entry into nerve axions or brain cells.

In the kinetic sense the ability to trace the path of a chemical reaction is most important if one wishes to know where most effectively to intervene in a reaction path, such as cholesterol synthesis or stopping cell lysis when salt or energy conditions, i.e. oxygen, change.

In order to measure the heats of reaction occurring in enzyme reactions, calorimeters are needed which can make measurements at the microcalorie level. The development of reliable batch and flow calorimeters for the study of biomolecular reactions has now reached a stage where even in a large 2 ml cell the rotational artifacts are only a few microcalories and are very reproducible. However, this system requires nearly 3 hours of equilibrium after loading so that only two experiments per day can really be done. Furthermore, such a long incubation time without stirring is not a good way to treat most biological samples, particularly cells. Gentle rocking can now be done and for continuous heat production systems such as cells this should make an excellent system.

During the past year, with the help of Dr. Theodor Kolobow, we have built a new all polypropylene flow cell. Molds for this cell have been made of aluminum in BEIB on a numerical mill. Casting under high temperature - high pressure conditions was then carried out in the BEIB plastics shop. Once a unit was made and flow tubes installed the unit could be inserted directly into the existing batch calorimeter. An interesting problem then developed almost immediately. Each time the solution was flowed, that is to say during the 20 seconds we flowed for example 50 microliters of each reagent into the mixer-reaction cell, a large endotherm reaction occurred. We then had to wait about ten minutes for the heat to return and equilibrium to be reached. This endotherm was caused by the evaporation of a very small amount of water through the flow cell wall. By coating this wall with a carbon coating laid down as a diamond lattice, no more than 2000 Angstroms thick, this effect was stopped. This coating was provided by a local company, Commonwealth Scientific, Inc., doing such work for the protection of computer disks. Electrical heater experiments indicate we can reproduce and detect 90 microjoules with a ten to one signal to noise ratio. This is equivalent to detecting the reacting of 2×10^{-9} moles of ATP breaking down to ADP. Flow artifacts are not more than the noise of the system when done differentially. All of the above figures apply to the differential mode. Very careful physical and thermal component matching is needed to achieve this level of common mode rejection both thermally and electronically.

A series of experiments are presently underway with Dr. Peter Simon, Department of Biochemistry, New Mexico University School of Medicine to determine the enthalpy of the reactions of spermine and aminacyl-tRMA synthetase with t-RNA. A theory of the transfer RNA specificity of the polyamines has been proposed by this group involving the release of water molecules in the transition state. They feel that a reliable enthalpy measurement will then tie down this hypothesis. However, problems with vibration noise, electrical and magnetic interference, and solution equilibrium at these extreme sensitivities must still be solved. The period from the time of introduction of the sample to the time when

equilibrium has been reached is less than one hour. Experiments can then be done repetitively every 10 to 15 minutes using as little as 25 microliters of sample or as much as 125 microliters. At this level of sensitivity a number of biological studies could be done. For example, in addition to a study of t-RNA polyamine specificity, the interaction of calcium with calmodulin and a variety of enzymes could be explored, almost all protein reactions will now be able to be studied thermodynamically.

This year has seen the completion of the differential pH-thermal automated titration apparatus. In addition to the EDTA-calcium reactions reported last year, true differential measurements have been carried out on EGTA-calcium and phytic acid-calcium chelation. Work, is presently underway on calmodulin-calcium titrations. We understand that a commercial instrument based on our design will be built by Commonwealth Technology, Inc.

Significance to Biomedical Research and the Program of the Institute:

The major thrust of biomedical research is to understand the basic physical and chemical processes that go on in cells and tissues, link these processes to the overall physiological functions of the living organism and thus better enable the practicing physician to keep the population healthy and more intelligently treat the patient when there is illness. The instruments being developed under this project will permit the biomedical investigator to determine the thermodynamic constants that are needed to develop a correct theory for the uptake and exchange of oxygen, carbon monoxide, carbon dioxide, etc. in the blood, tissues, and lungs. Calcium is an important regulator of cellular function and the constants which will be determined with these instruments will ultimately lead to a better understanding of how this regulation takes place. The manner in which enzymes work with DNA to do what the cells needs to do for synthesis is one of the fundamental problems of molecular biology and just as in organic chemistry, the details of these reactions must be known if they are ultimately to be used in the vast number of genetic applications now being started in this rapidly growing new field.

Proposed Course:

The batch calorimeter, 2 ml cell, will be instrumented for use with cells. Optical pH, pCO_2, and pO_2 probes will be introduced along with lines for various titrants to add substrate and inhibitors. We will thus be able to run the system as a Warbug respirometer but with 10^3 to 10^4 fold increased sensitivity. The flow calorimeter will be further improved to eliminate all artifacts and noise.

Publications:

Berger, R. L., Cascio, H. E., Davids, N., Gibson, C. G., Marini, M., and Thiebault, L. J.: J. Biochem. & Biophys. Methods, in press, 1984.

David, N., Berger, R. L., and Marini, M.: J. Biochem. & Biophys. Method, in press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 01421-09 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Electrochemical and Physiological Methods for Cell Research

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. E. Steele	Physical Science Investigator	LTD:NHLBI
Others:	R. L. Bowman	Chief, LTD	LTD:NHLBI
	A. P. Tegtmeyer	Chemist	LTD:NHLBI
	J. W. Handler	Section Chief	KE:NHLBI
	W. Haller	Chief, Inorganic Glass	NBS

COOPERATING UNITS *(if any)*

Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The porous bottom culture dishes (PBCDs) and the devices for the sterile measurement of the electrophysiological parameter of cell layers which we developed are receiving wide application. In addition to the studies of kidney epithelial here at NIH and in more than 50 other laboratories in the U.S.A. and abroad, they are being successfully used in the study of pulmonary epithelia. We have shown that the electrophysiological responses A6 (Xenopus laevis kidney) cell layers are enhanced by increasing the exchange of nutrients and wastes at both the upper and lower surfaces by gently shaking the PBCD's in the incubator during the entire development of the cell layer and its Na transport capability.

The major role played by Ca^{++} in the regulation of transport and other process in cells has prompted us to improve the methods of measuring Ca^{++} activity inside epithelial cells of many kinds. Microelectrodes need to be 0.1 or 0.2 microns in diameter to enter most epithelial cells without causing damage which makes reliable readings impossible. It is logically argued that electrodes of this size have walls so thin that they are totally hydrated near the tip and with the monovalent ions of the glass become Na^+ and k^+ electrodes. This conflict between Ca^{++}, Na^+ and K^+ sensitivities is believed to cause the poor performance often seen for electrodes smaller than 1 micron in diameter at Ca^{++} activities below 10^{-6} molal. We have therefore designed, constructed, and used unique anular burners for making micropipettes form fused quartz. These fused quartz micropipettes are made from high purity material so that no metal ions will be present in the wall to give the completed microelectrodes unwanted ionic responses. It is expected that this will significantly improve the accuracy of intracellular Ca^{++} activity measurements of epithelial cells.

1042

Project Description:

Objectives:

(1) Develop instruments and methods to make possible the study of cells in culture in ways that have not been done before (i.e. our PBCDs for the study of epithelial cells in culture).

(2) Improve methods for the measurement of intracellular Ca^{++} activity of individual cells. Measurements in epithelial and endothelial cells in culture are our prime objective.

Methods and Results:

1. Using our porous bottom culture dishes (PBCDs) and devices for sterile measurement of potential difference, short circuit current and resistance of epithelia grown on these PBCD's, we have studied the effect of increasing the exchange of nutrients and wastes at the cell surfaces by gently shaking the PBCD which have 1 mm feet during the entire growth and differentiation period of A6 (Xenopus lalvis kidney) cells in the incubator. The shake was a 5 mm excursion once per second obtained by using a vacuum driven windshield wiper motor to push an incubator shelf section back and forth on Teflon guides. The sodium transport (as indicated by short circuit current) was found to be larger for the shaken preparation compared to still controls at all times. This difference became more pronounced as the preparations became older. At 27 days after placing the cells on the PBCD's the transport of the shaken group exceeded the controls by a factor of 3 or 4. This ratio resulted primarily from the decline in transport of the still group just as we have commonly observed. Thus the shaking produces a nearly stable transport over a period of about a month. The percentage response to vasopressin addition by the two groups was about the same when the short circuit current was observed. However, when the electrical resistance decrease which follows vasopressin addition was calculated, the shaking group had a resistance decrease 2 times as great as the still controls. Thus the shaking yields preparations with nearly stable transport for a period of a month and a vasopressin induced resistance decrease which is much greater than found in still controls.

2. We have designed, constructed and used a unique anular burner to make micropipettes from fused quartz (pure silica). These micropipettes are for use in microelectrodes smaller than 0.3 microns tip diameter for the intracellular measurement of Ca^{++} activity in epithelial and endothelia cells. The use of pure silica is expected to eliminate erroneous responses to ions other than Ca^{++} due to the movement of ions through the walls of conventional pyrex micropipettes when they are made very small for entry into small cells.

Several designs of our anular burner have been made and tested in two commercial micropipette pullers by replacing the electrical heating coil or loop with one of our anular burners. The burners are small enough to fit in the available space. By carefully controlling the pressure (and flow) of the gas (propane) and oxygen, the centering and the timing of the flame and pulling forces, reproducible pipettes can be produced. Tubing ranging from 0.1 mm to 2 mm has pulled used successfully. Most have been pulled from 1.0 mm O.D., 0.8 mm I.D. fused quartz tubes. The next step is to silanize these micropipettes in a flow through system. This method is expected to more reproducible coatings than the conventional "beaker in a oven" method. It should also allow the use of longer chain silanizes without decomposition by providing better temperature and gas control.

Significance to Biomedical Research and the Program of the Institute:

The apparatus and method necessary to grow and study sheets of epithelial cells on membranes is advancing the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile conditions greatly increases the productivity of this work. Dr. Handler, LKEM, NHLBI, is presenting the physiological significance of this work in detail. The growth of the cells on nutrient and gas permeable membranes results in a degree of development and differentiation which does not occur on the conventional plastic or glass surface. This should allow a study of the development of epithelial that is not possible otherwise. The accurate measurement of the Ca^{++} activity inside the epithelial cells at various stages of transport development should greatly increase our knowledge of transport development and control.

Proposed Course:

Design sterile continuous flow apparatus for sterile study of sheets of epithelial cells on transparent membranes for weeks or even months so that the development of transport and morphology can be followed.

2. Develop defined media for epithelial cells which yields optimal differentiation. This may require 2 media: one for a growth period and one for differentiation.

   Develop and try techniques for growing two different types of cells on the two sides of the PBCD membranes.

   Develop electrodes to measure Ca^{++} activity in epithelial and endothelial cells which have tips smaller than 0.3 microns and low interferences so that Ca^{++} activities of 10^{-6}, 10^{-7}, 10^{-8} and even 10^{-9} molal can be measured correctly.

a. Silanize pipettes in flow through system with long chain monochlorosilanes.

b. Coat outer surface of microelectrode in such a way as to facilitate entry into cells.

Publications:

1. Handler, J. S., Preston, A. S., and Steele, R. E.: Factors affecting the differentiation of epithelial transport and responsiveness to hormones. Federation Proc. 43: 2221-2224, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01435-05 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

New Catheter Idea to Facilitate Radiologic Instrumentation

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. L. Bowman	Chief, LTD	LTD:NHLBI
Others:	R. Paul	Phys. Sci. Techn.	LTD:NHLBI
	J. Doppman	Chief, Radiology Dept.	DR:CC

COOPERATING UNITS *(if any)*

Diagnostic Radiology Department, Clinical Center, NIH

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The concept of a topocatheter extension added to a standard angiographic catheter involves the production of a high strength thin walled tapered tube attached to the end of a standard catheter. Suction on the catheter causes the thin wall portion to collapse and invert itself until it lines the distal end thin wall portion to collapse and invert itself until it lines the distal end of the catheter. Flow out of the catheter causes the thin wall to valve shut and evert again. When the thin wall everts it rolls onto the inside of the vessel wall with no relative wall motion hence no friction. The rolling wall is advanced by the pressure of the fluid inside so that the extension enters and follows small or tortuous vessels with ease.

Catheter extensions have now been made of reconstituted collagen sealed with a polyurethane dip coat to improve the strength and compliance to inversion and eversion while reducing the possibility of blocking blood flow by balooning. extensions up to 15 cm long on number 5F have successfully entered cerebral vasculature in the monkey.

Testing of these catheters have shown them to require 900 mm of Hg rupture them but eversion is accomplished below 200 mm of Hg. Tests of other collagens have indicated that our present methods are adequate and EM studies confirm a multilayer interwoven fibrous structure and good adherence to the polyurethane.

Application to the therapy of AV malformations in the Radiology Department is anticipated.

1046

Project Description:

Objectives:

To improve the strength and performance of the extension everting angiographic catheters and characterize the performance specifications required for safe human therapeutic applications.

Methods Employed:

We have continued to test other formulations of collagen and other polyurethanes using the established method which uses multiple coats of collagen applied to rotating glass forms and finally dip coating in solutions of polyurethanes.

A modification that appears to impart more flexibility and some increase in optical clarity without loss of strength was accomplished by the addition of about 5% kangaroo tail collagen added to the usual bovine hoof collagen provided by Ethicon. A collagen provided by the Helitrix Corporation had no strength.

A system for recording the pressure necessary to produce eversion and bursting pressure was assembled and catheters were tested for uniformity and pressure tolerance. Most catheters everted at about 200 mm Hg but took about 900 mm Hg to rupture them with the tip occluded. These values apply to a #5 French size. Larger catheters can be made thicker to retain the performance of the smaller ones.

The structure of the catheters was examined by scanning and transmission electron microscopy with the cooperation of Dr. Walker Jones of the Zoology Department at Howard University who has some interest in our collagen tubes for application to endothelial cell culture research. The examination confirmed that our method of multiple coats applied in alternating bias directions does make a tear resistant structure of overlaping fibrous layers. Pontamine Blue dye was found to be an excellent dye for staining the collagen fibers. In addition the good bond between the collagen and the polyurethane was confirmed.

Flow visualization studies were recorded using the model of the cerebral circulation built by BEIB which confirmed the ability of these catheters to deliver high flows without distention and demonstrated their flexibility and tolerance to the pulsations. Some modifications to the ends of the catheter were demonstrated to provide a means of increasing the mixing of the therapeutic agent with the blood stream.

Major Findings:

We have demonstrated that collagen-polyurethane combination catheters can be made to function with infrequent failure in sizes down to #4 French in

lengths adequate to reach any major vessel in the brain without requiring the carrier catheter to traverse the intricate curves of the carotid siphon.

The therapeutic experience with the treatment of the glioblastomas has had some poor results presumably due to streaming of the therapeutic agent so that the emphasis in the future will be toward the treatment of cerebrovascular malformations. As these problems present a special problem for each particular patient it appears that the flexibility of our methods will allow us to custom make the catheters required for each specific problem.

Significance to Biomedical Research and the Program of the Institute:

The art of therapeutic interventional radiology has opened new approaches in intravascular therapy. The extension catheter reaches beyond the range of angiographic catheters to reach small vessels via tortuous channels.

The everting extension is made of very thin-walled collagen and is tapered slightly to permit entry into smaller vessels than the supporting angiographic catheter.

The tapered end everts into small vessels without wall friction and the very thin walls allow the extension to flatten against the vessel wall so that blood flow is little compromised by its presence. During infusion of therapeutic agent the thin walls do not produce any back pressure at any flow that can be produced through the angiographic catheter that it is attached to.

The present application to the carotid makes it imperative that the flow does not distend the thin walls and block blood flow. This non-distensibility of the collagen, the very thin walls and the ample lumen suggest that these same qualities would be desirable for entering the coronary vessels to deliver a therapeutic agent beyond a constricted lumen.

Proposed Course:

We are continuing to improve the methods of forming and coating these catheters to further reduce defects and to explore special shapes. In changing our objectives toward therapy of cerebrovasular malformation rather than tumor therapy we will concentrate on our capacity to tailor make an optimal catheter for a specific therapeutic challenge. Preliminary tests on triangular cross section mandrils and newer polyurethane rubber are promising and will be further developed. publication of the method awaits further clinical results.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01445-03 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Continuous Development of High-Speed Preparative Countercurrent Chromatography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Y. Ito	Senior Investigator	LTD:NHLBI
	J. L. Sandlin	Biologist	LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.3	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The efforts have been further continued to increase the preparative capability of the high-speed countercurrent chromatography (HSCCC) with a large diameter coil. The apparatus is a bench top model of the horizontal flow-through coil planet centrifuge which produces the suitable synchronous planetary motion of the column holder at 15 cm revolutional radius. The separation column was prepared from a piece of 5.5 mm i.d. FEP tubing which was coaxially coiled around the holder. The short column for preliminary separations was prepared from 4.2 m long, 112 ml capacity tubing and the long multilayer coil for preparative separations, from a 30 m long, 750 ml capacity tubing. The performance of the present scheme was assessed with a standard set of dinitrophenyl (DNP) amino acid samples and a two-phase solvent system composed of chloroform, acetic acid, and 0.1N hydrochloric acid at a volume ratio of 2:2:1.

A series of experiments was performed to study the effects of revolutional speed (50 rpm - 400 rpm), flow rate (120 ml/h and 500 ml/h) and helical diameter of the column (7.5 cm, 11.25 cm, and 15 cm) on the retention of the stationary phase and peak resolution. Results showed that both the revolutional speed and the helical diameter plays a critical role in hydrodynamic distribution of the two solvent phases in the coil. With the proper mode of elution, a large amount of the stationary phase was retained in the coil resulting in excellent peak resolution even under a high flow rate of 500 ml/h. Large-scale preparative separations with 20 ml sample volume have been successfully demonstrated with the multilayer coil.

1049

Project Description

Objectives: Development of high-speed countercurrent chromatography (HSCCC) with a large preparative capacity.

Methods Employed:

A. Principle

The principle of HSCCC has been described earlier in Z01 HL 02445-02 LTD.

B. Apparatus

A bench top model of the horizontal flow-through coil planet centrifuge was used. The apparatus holds a pair of holders symmetrically at a distance of 15cm from the centrifuge axis. Each holder is equipped with a planetary gear which is engaged to the identical sun gear mounted around the central stationary pipe. This gear arrangement produces a desired synchronous planetary motion to the holders, i.e., rotation and revolution at the same angular velocity and in the same direction. In the present study, the column was mounted on one of the holders while the other holder was used for mounting the counterweight. The column holder is made removable from the rotary frame to ease winding the column on the holder. The short columns were prepared from a 4.2 m long piece of 5.5 mm i.d. FEP tubing, 112 ml capacity, by winding it coaxially around the holder making uniform helical turns. Three columns with different helical diameters of 7.5 cm (β=0.25), 11.25 cm (β=0.375), and 15 cm (β=0.5), were prepared by choosing the respective core diameters of the holder, where β is the ratio of the coil radius to the orbital radius of the holder. The long preparative column was prepared from a 30 m long piece of the same tubing, 750 ml capacity, by winding it around the holder making multiple layers of the coil. The revolutional speed of the apparatus was continuously adjustable up to 400 rpm with a speed control unit (Bodine Electric Co.).

C) Separation Procedure

The two-phase solvent system was prepared by mixing chloroform, acetic acid, and 0.1N hydrochloric acid at a 2:2:1 volume ratio in a separatory funnel at room temperature. The sample solutions were prepared by dissolving a mixture of DNP amino acids in the upper and/or lower phase.

In each separation the column was first filled with the stationary phase followed by injection of the sample solution through the sample port, Then the apparatus was run at the desired revolutional speed while the mobile phase was pumped into the column at a given flow rate. The elution was performed by using both the upper and the lower phases as the mobile phase each in two different modes, i.e, head to tail and tail to head through the

column. The effluent through the outlet of the column was continuously monitored with an LKB Uvicord S at 280 nm and then fractionated into test tubes for determination of absorbance at 430 nm with a Beckman DU spectrophotometer. After the centrifuge run was completed, the column inlet was connected to a N_2 gas line (80 psi) and the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column.

Major Findings:

The effects of β value on the retention and partition efficiency were investigated by using three different helical diameters. The results revealed that the hydrodynamic trend of the two solvent phases is largely affected by the β values. At β = 0.25 (7.5 cm helical diameter), the lower nonaqueous phase was unilaterally distributed toward the head of the coil and the upper aqueous phase toward the tail of the coil. At β = 0.5 (15 cm helical diameter), the above hydrodynamic trend of the two solvent phases were completely reversed, and the upper phase was distributed toward the head and the lower phase toward the tail. In either case the experimental runs have produced excellent retention and good peak resolution at 300 rpm and at a high flow rate of 500/ml/h under the proper mode of elution. When β = 0.375 (11.25 cm helical diameter), however, two solvent phases showed an unstable transitional hydrodynamic trend and failed to yield satisfactory retention levels of the stationary phase even at a slow flow rate of 120 ml/h.

Under the best operational conditions determined by the preliminary runs with the short columns, large-scale preparative separations were performed with a long multilayer coil of 750 ml capacity mounted at the two different positions, one with a small β value near 0.25 and the other with a large β value near 0.5. Both columns produced satisfactory separations of five DNP amino acid samples with high retention levels of the stationary phase of over 70%. Comparison between the results obtained from these two β values revealed that the small β value produced substantially higher partition efficiencies while the large β value yielded higher retention level of the stationary phase. In short the present scheme is capable of producing efficient separations of gram quantities of samples in short periods of time. The results also suggest that the present scheme can be further scaled up by using the column with a larger and/or longer tubing.

Significance to Biomedical Research and the Program of the Institute:

Separation and purification of biological samples are essential for most of the biomedical studies. CCC offers an advantage over other chromatographic methods in that all complications arising from the use of solid supports are entirely eliminated. The present scheme is capable of separating gram quantities of samples in short periods of time.

Proposed Course:

1. Application of the method to various two-phase solvent systems.

2. Further increase of sample-loading capacity by the use of a larger-diameter and/or longer column.

Publications:

Sandlin, J. L. and Ito, Y.: Gram quantity separation of DNP amino acids with multi-layer coil countercurrent chromatography (CCC), J. Liq. Chromatogr. Vol. 7, No. 2 (1984) 323-340.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01449-02 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Monolithic Integrated Countercurrent Chromatography (MICCC)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Kolobow	Medical Officer	LTD:NHLBI
	Y. Ito	Medical Officer	LTD:NHLBI
Others:	I. Mychkovsky	Phys. Sci. Aid	LTD:NHLBI
	J. Morabito	Biol. Lab. Techn.	LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1½	½	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have designed a monolithic integrated countercurrent chromatography (MICCC) system for the continuous or batch separation of solutes, particles, or cells. We are using differential solubility of compounds in a two phase solvent system as the principle in chemical compound separation. The MICCC system represents a multistage system embodying this principle, and is formed of a suitable plastic, or metal. Separation is enhanced when MICCC is vibrated, shaken, or otherwise agitated for enhanced mixing.

The centrifugal MICCC was devised to greatly increase the capacity of MICCC by providing improved mixing, and excellent phase retention. Such a device can be particularly useful for particle, and cell separation.

1053

Project Description

Objectives:

It is the purpose of this project to devise new integrated systems for the separation of solutes, or particulate biological structures, either continuously, or in batch form. Such methods could result in cleaner separation of biologic compounds and structures, and may lay the foundation for future advances.

Methods Employed:

We have employed methods based on differences in interfacial forces, surface charge, buoyancy, and in solubility, in devising an integrally molded system (monolithic integrated countercurrent chromatography = MICCC).

This year, we have mainly explored methods that effect separation of solutes based on differential solubility of compounds in a two phase solvent system chosen so that desired compounds will appropriately and optimally partition. To devise a compact separation chamber, we have formed through a novel molding technique an integrated flow channel consisting of numerous locules interconnected with each other. The locules, and flow channels, can be varied in dimension and in orientation. To effect mixing, the molded plastic sheet with the embossed MICCC is placed on a frame and subjected to various forms of motion to effect enhancement in mixing, and thus improve on separation. We have explored oscillatory motion and gyrational motion.

For substantially more rapid separation at substantially faster flow rates, we have constructed a centrifugal MICCC system. The enhanced gravitational field allows for exceptionally high retention, greatly improving on resolution in the process of solute separation. The centrifugal MICCC also appears to be well suited to particle and cell separation.

Major Findings:

We have now built and tested a MICCC system made of polypropylene, embossed with up to 2,500 separate compartments. In tests using DNP alanine and DNP glutamic acid, we were able to achieve separation equivalent to over 1000 theoretical plates.

On a practical basis, it is more useful to improve on resolution by reducing the total number of individual compartments, and thereby to increase total volume. MICCC systems with total number of compartments limited to between 100 and 600 were found to be especially useful.

We find that externally applied motion (gyrational, oscillational) greatly enhance equilibration between the two solvent systems, and thus on resolution. A frequency of 10 Hz appears to be optimal to effect phase equilibration as well as stationary phase retention.

Limitations found in MICCC due to loss of stationary phase are minimized in the centrifugal MICCC model. We have noted excellent retention of the stationary phase in the centrifugal MICCC, even at flow rates an order or more greater in magnitude compared to the MICCC system. No data are as yet available as to the efficiency and resolution of the centrifugal MICCC.

Significance to Biomedical Research and the Program of the Institute:

Enhanced means of chemical separation without structural alterations by and from the separation process can provide us the means to obtain ultrapure compounds in large quantities. Such availability would be of help in yielding compounds devoid of extraneous effects, and help us in their characterization. The frontiers of knowledge, and clinical applications, will both be significantly enhanced. In addition, the centrifugal MICCC is likely to be useful in particle separation as well. A high throughput can yield a large quantity of a desired cell population, when using polymer phase solvent systems.

Proposed Course:

We will continue work on devising a practical MICCC system, and the centrifugal MICCC for both solute, and biological particle, and cell separation. We will explore variables to allow for their optimal construction and performance.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01450-01 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less Title must fit on one line between the borders)*

Studies on hydrodynamics in High-Speed Countercurrent Chromatography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Y. Ito Senior Investigator LTD:NHLBI

COOPERATING UNITS *(if any)*

Department of Pharmaceutics, School of Pharmacy, State University of New York, at Buffalo (Dr. Walter D. Conway).

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Development of high-speed countercurrent chromatography requires basic knowledge of hydrodynamic behavior of the two-phase solvent system. With several types of the horizontal flow-through coil planet centrifuge devices, motion and distribution of two immiscible solvent phases in coiled columns were studied under various experimental conditions.

Results obtained with fifteen different types of commonly used solvent systems were summarized below:

1) Under a proper mode of synchronous planetary motion, the two solvent phases are unilaterally distributed in the rotating coil with one phase (head phase) on the head side and the other phase (tail phase) on the tail side.

2) Three solvent groups classified according to the hydrophobicity of the non-aqueous phase display the characteristic hydrodynamic trend: In the hydrophobic solvent group the upper phase becomes the head phase, while in the hydrophilic solvent group the lower phase becomes the head phase. In the intermediate solvent group, the head phase is determined by a parameter beta which is the ratio of the coil radius to the orbital radius of the column holder.

3) The above hydrodynamic trend of the solvent system is most closely correlated to viscosity of the solvent system so that raising the temperature results in drastic change of the hydrodynamic trend of the hydrodaynamic trend of the solvent system especially in viscous butanol solvent systems.

4) Stroboscopic observation of the colored solvent phases revealed that each coiled turn contains mixing and settling zones, each zone traveling toward the had end of the coil at a rate of the revolution speed of the coil. This indicates that solutes present in the coil are subjected to a repetitive mixing and settling process in the coil at an enormously high rate of over 13 times a second which explains the high partition efficiency produced by high-speed countercurrent chromatography.

1056

Project Description

Objectives:

Experimental studies on motion and distribution of the two-phase solvent system in high-speed countercurrent chromatography (HSCCC). The studies are divided into four parts as follows:

Part I: Hydrodynamic distribution of two solvent phases in a helical column by two different modes of synchronous planetary motion.
Part II: Phase distribution of a set of conventional solvent systems in helical and spiral columns under various experimental conditions.
Part III: Effects of temperature on the hydrodynamic distribution of the solvent system.
Part IV: Stroboscopic observation of the colored solvent phases in the rotating coiled column.

Methods Employed and Major Findings:

Part I: A combined horizontal flow-through coil planet centrifuge was constructed and used in the present study. The apparatus holds a pair of coil holders, each providing a different mode of planetary motion. The pulley-driven holder (scheme I) synchronously counterrotates about its own axis while revolving around the central axis of the centrifuge. The gear-driven holder (scheme IV used for HSCCC) undergoes similar synchronous planetary motion except that the rotation and the revolution of the holder are in the same direction. A helical column was prepared from a piece of PTFE tubing, i.6 mm i.d. and 5 m long, by winding it onto the holder making uniform helical turns of 10cm diameter. In each experiment the column was entirely filled with the stationary phase and the mobile phase was pumped into the column at a given flow rate while the apparatus was rotated at a desired revolutional speed. The effluent from the outlet of the column was collected into a graduated cylinder to measure displacement of the stationary phase for computation of the volume retained in the column. The run was performed by using both the aqueous and nonaqueous phases as the mobile phase. Percentage retention relative to the total column capacity was plotted against the applied revolutional speed in rpm to produce the phase distribution diagram which indicates retention profile and hydrodynamic trend of the solvent system in the rotating column.

The phase distribution diagrams obtained from nine conventional two-phase solvent systems revealed a drastic contrast between the two modes of the synchronous planetary motion of the holder. In the scheme I planetary motion (pulley drive) the two solvent phases establish a basic hydrodynamic equilibrium which produces near even distribution of the two phases on the head side of the coil, resulting in low retention levels of the stationary phase. In the scheme IV planetary motion (gear drive used for HSCCC) the two phases establish unilateral hydrodynamic distribution where one phase

is directed toward the head and the other phase toward the tail in the coil, yielding satisfactory retention levels of the stationary phase under a proper mode of elution.

Part II: Phase distribution in helical and spiral columns obtained from the combination of various revolutional radii and holder diameters.

Three different models of the horizontal flow-through coil planet centrifuges (scheme IV synchronous planetary motion) provided specific range of the revolutional radius for the given set of holders having different diameters. Mathematical analysis of the acceleration produced by the scheme IV planetary motion indicated that a parameter $\beta = r/R$, where r is the coil radius and R, the orbital radius of the column holder, may play a key role in hydrodynamic phase distribution in HSCCC.

The two types of column configuration were employed. The helical column (1.6 mm i.d.) was prepared by winding the tubing onto the holder as described in Part I. The spiral columns (1.6 mm i.d. and 2.6 mm i.d.) were prepared by winding the tubing tightly between a pair of closely spaced flanges mounted around the holder to make a single-layer flat spiral configuration.

Measurement of the phase retention was performed according to the procedure described in Part I. In the helical column, elution was performed in two mobile phase. In the spiral columns which provide the internal and external terminals, elution was performed in four different modes for each mobile phase, i.e., the internal head to the external tail, the internal tail to the external head, the external head to the internal tail, and the external tail to the internal head. The set of retention data obtained from the different elution modes of the single mobile phase was expressed in the same phase distribution diagram with the respective symbolic designs of the retention curves to facilitate the comparison.

The phase distribution diagrams for the helical column obtained from fifteen different types of the two-phase solvent systems revealed characteristic hydrodynamic trends of the solvent phases according to the hydrophobicity of nonaqueous phase. The hydrophobic solvent group including hexane/water, ethyl acetate/water, and chloroform/water, yielded excellent retention levels regardless of the applied range of β values and exhibited a hydrodynamic trend where the upper phase is always distributed toward the head of the coil and the lower phase toward the tail of the coil. The hydrophilic solvent group, which includes n-butanol/acetic acid/water (4:1:5) and sec.-butanol/water, yielded satisfactory levels of retention but exhibited a reversed hydrodynamic trend where the lower phase is distributed toward the head and the upper phase toward the tail. The intermediate solvent groups characterized by moderate hydrophobicity of the nonaqueous phases includes the rest of the solvent systems such as

hexane/methanol, ethyl acetate/acetic acid/water (4:1:4), chloroform/acetic acid/water (2:2:1), and n-butanol/water. These solvent systems showed variable hydrodynamic trends where the lower phase becomes the head phase in small β value (0.125) and the upper phase becomes the head phase in the large β value (0.75). Addition of NaCl at a 1M concentration to the hydrophilic solvent systems altered their hydrodynamic trends into those in the intermediate solvent systems.

The phase distribution diagrams for the spiral columns clearly illustrated effects of two different forces, one produced by the Archimedean screw effect determined by the head-tail relationship of the coil and the other produced by the centrifugal force gradient acting from the internal terminal toward the external terminal of the spiral column. In the hydrophobic solvent group, the effects of the force gradient was trivial while in the hydrophilic solvent group it gave considerable influence on the retention profile of the solvent system especially in a large-bore column with large β values. However, the force gradient by itself failed to provide the satisfactory retention levels under the applied operational conditions. Therefore, it is concluded that the force gradient produced by the spiral configuration of the column is less important than the head-tail orientation of the coil, in retention of the stationary phase in HSCCC.

Part III. Correlation of physical properties of the solvent system to the hydrodynamic trend and beneficial effects of temperature on HSCCC.

Three major physical properties of the two-phase solvent system, i.e., interfacial tension, viscosity, and density were measured with the conventional methods and the values were used for the correlation studies. The hydrodynamic trend of the solvent system in the centrifugal force field was found to be closely correlated with the settling times of the solvent phases in the unit gravity. The hydrophobic solvent group, in which the upper phase is the head phase, showed the shortest settling times ranging from 1 sec to 15 sec, while the hydrophilic solvent group, in which the lower phase is the head phase, gave the longest range from 30 sec to 60 sec. The intermediate solvent group produced moderate settling times between 15 and 30 sec. Consequently, the settling times provide a reliable numerical expression for the hydrodynamic trend of the solvent system which can be conveniently used for computation of the correlation coefficient (r) against the physical properties of the solvent system.

Among three physical properties of the solvent system, viscosity was found to have a strong correlation (r= + 0.88) to the settling times indicating that the less viscous the solvent phases, the higher level of retention is expected. Interfacial tension (r =-0.65) and the density difference (r=-0.45) between the upper and the lower phases gave moderate to weak correlations, respectively.

Because viscosity of the solvent system is sensitively affected by temperature, raising the temperature above room temperature would improve the retention of the stationary phase for viscous hydrophilic solvent systems. This possibility was tested by measuring the settling times of the solvent system under temperatures ranging from 20°C to 80°C. Binary solvent systems such as n-butanol/water and sec.-butanol/water showed smooth exponential decline of the settling times with increased temperature, reaching below 10 sec and 20 sec, respectively, at 50°C. In the ternary system of n-butanol/acetic acid/water (4:1:5), the settling times declined with the increase of the temperature up to near 50°C reaching the lowest level of 23 sec. Further increase of the temperature resulted in increase of the settling times until it reached 65°C where the solvent formed a single phase. This increase in settling times is most likely due to the extremely low interfacial tension and small density difference between the two phases near the plait point of the solvent system. This problem can be eliminated by either decreasing the amount of acetic acid or adding the salt to the solvent system.

Performing HSCCC under the elevated temperature conditions will provide a number of beneficial effects such as 1) conversion of the hydrodynamic trend of the hydrophilic solvent systems to the normal mode, 2) improved retention of the stationary phase 3) higher partition efficiency due to reduction of mass transfer resistance, 4) increased sample-loading capacity due to higher solubility, etc. Recently, a HSCCC apparatus equipped with a temperature control system has been constructed in our laboratory. The preliminary test runs have produced highly promising results.

Part IV: Stroboscopic observation on hydrodynamic motion of the colored solvent phases in spiral and multilayer helical columns in the HSCCC centrifuge.

The study was performed by a horizontal flow-through coil planet centrifuge equipped with a transparent plastic coil holder mounted at a distance of 10 cm from the centrifuge axis. The single-layer spiral column consisted of about 3.5 m of 2.6 mm i.d. PTFE tubing, volume 21 ml, with 10 coils from 11.5 cm (β = 0.57) to 17 cm (β = 0.9) in diameter. The multilayer coil consisted of about 17 m of the same tubing, volume 102 ml, 5 rows wide, 10 helical layers ranging from 10 cm (β = 0.5) to 17 cm (β = 0.85) in diameter. The solvent system was composed of chloroform, acetic acid, and water at a 2:2:1 volume ratio and equilibrated in a separatory funnel at room temperature. The column was first filled with the stationary phase and the mobile phase pumped at 240 ml/h into either end while rotating the apparatus at 800 rpm. A stroboscope was magnetically synchronized for observation and photography of the phases dyed yellow and red.

Hydrodynamic phase equilibrium was quickly established behind the mobile phase front. The best retention was obtained with the column head at the

center and lower phase pumped from head to tail (82%) or upper phase pumped from tail to head (84%). However, only slightly lower retention (78% and 77%) was obtained with reverse rotation.

Under condition with good retention, the column was divided into an agitated zone, a one quarter helical turn near the center of the centrifuge, in which droplet formation and good mixing were observed and a quiescent zone in the remainder of the column where the lower phase migrated along the periphery of each coil with little apparent mixing. This finding indicates that the mixing zone moves constantly toward the head of the coil at a rate of revolution and solutes present in the coil are subjected to a repetitive mixing and settling process at an enormously high rate of over 13 times a second, thus explaining high partition efficiencies produced by HSCCC.

Significance to Biomedical Research and Program of the Institute:

Separation and purification are important first steps in many biomedial studies. CCC yields excellent sample recovery and high purity fractions. HSCCC offers a number of advantages over other CCC schemes such as short separation times, good stationary phase retention, high peak resolution, large sample-loading capacity, etc. The present studies on hydrodynamic phase distribution would greatly facilitate further development and refinement of HSCCC as well as the applications of the method to various biomedical samples.

Proposed Course:

Hydrodynamic studies with the HSCCC centrifuge equipped with a temperature control system.

publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01451-01 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Electron Spin Resonance Development for Medical and Biological Problems

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator) (Name, title, laboretory, and institute affiliation)*

PI: J. Zweier Staff Fellow LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to develop and adapt Electron Spin Resonance Spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. To accomplish this goal we are working on approaches to increase the sensitivity and developing cavity design suitable for different problems ranging from microsamples, to cultured cells to whole tissues.

Over the past year an X-band spectrometer has been assembled. A technique was developed for calculating intramolecular distances using Electron Spin Resonance, and it was applied to measure the distance between the 2 metal binding sites of the iron carrier protein transferrin a critical question in the field of iron metabolism. This technique is currently being applied to determine the location of receptor proteins on the cell plasma membrane or within the cell cytoplasm. Electron spin resonance has a major advantage over the conventional morphologic and cell fractionation techniques in that the integrity of the cell is maintained.

The important anticancer drug adriamycin was also studied to determine how it kills tumor cells and how it induces a cardiomyopathy, which is the third most common cardiomyopathy in the United States. It was demonstrated that Fe(III) binds to adriamycin and that these complexes then cycle to reduce oxygen. The Fe(III) bound to adriamycin is reduced to Fe(II) which then donates an electron to oxygen, regenerating Fe(III) - adriamycin. Numerous studies have shown that the therapeutic and toxic effects of adriamycin are due to the formation of reactive oxygen radicals and this mechanism provides an explanation of how these radicals are formed.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description

Objectives:

The overall goal of this project is to develop and adapt Electron Spin Resonance Spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. Electron Spin Resonance Spectroscopy is a powerful technique for studying metal metabolism and free radical generation. For paramagnetic metal ions such as Fe(III), Cu(II), and Mn(II, Co(II) information can be obtained about the redox state of the metal, the spin state, the symmetry of metal binding and the identity of binding ligands. For free radicals information can be obtained about their structure, mobility, stability, and quantitation. The Electron Spin Resonance technique provides critical information on these problems which can not be obtained in any other way. It has been applied to a large number of biological problems at the in vitro biochemical level, however, the great potential of this technique has not been fully realized due to limitations in the sensitivity and sample geometry of conventional commercial spectrometers. With conventional commercial spectrometers only samples in 1-3 mm tubes or flat cells can be studied and the sensitivity is limited to 10^{-4} - 10^{-5} M concentrations of metal ions such as Fe(III) or Cu(II) or 10^{-6} M concentrations of free radicals. In order to study in vivo metal metabolism and free radical generation in intact cells and tissues it is first necessary to be able to accommodate the geometries of these biological samples and then the sensitivity must be optimized in order to be able to detect the concentrations of metals and radicals found within cells. Therefore, we are working on approaches to increase the sensitivity and developing spectrometer and cavity designs suitable for a large range of biological problems ranging from microsamples, to cultured cells, to whole tissues. While developing these techniques we are demonstrating their feasibility and importance by actively applying them to solve important medical and biological problems.

Methods Employed:

Over the past year an X-band Electron Spin Resonance Spectrometer has been assembled complete with variable temperature capability from 4°K to 40°C. Work was performed evaluating a number of different cavities and resonators including rectangular TE cavities, cylindrical TE and TM cavities and loop gap resonators.

Major Findings:

A technique was developed for calculating intramolecular distances using Electron Spin Resonance and it was then applied to measure the distance between the two metal binding sites of the iron transport protein transferrin, a critical question in the field of iron metabolism. A

A distance of 41.6 ± 2.8Å was obtained and this is the first magnetic resonance measurment of the distance between transferrin's two sites (Zweier, J. Biol. Chem. 258, 1984). With this technique distances between two paramagnetic ions are calculated from the paramagnetic broadening in the signal of one ion from the other. These measurements can serve to complement and confirm fluoresence energy transfer measurements of inter and intra molecular distances. This technique is currently being applied to determine the location of receptor proteins on the cell plasma membrane or within the cell cytoplasm. Electron Spin Resonance has a major advantage over the conventional morphologic and cell fractionation techniques in that it is nondestructive and the integrity of the cell is maintained.

The important anticancer drug adriamycin was also studied to determine how it kills tumor cells and how it induces a cardiomyopathy, which is the third most common cardiomyopathy in the United States. It was demonstrated that Fe(III) binds to adriamycin and that these complexes then cycle to reduce molecular oxygen. The Fe(III) bound to adriamycin is reduced to FE(II) which then donates an electron to molecular oxygen, regenerating Fe(III) - adriamycin (Zweier, 1984 J. Biol Chem, in press). The formation of an oxidized adriamycin radical was also observed. Numerous studies have shown that the therapeutic and toxic effects of adriamycin are due to the formation of reactive oxygen radicals and the cycle of iron mediated oxygen reduction provides an explanation of how these radicals are formed.

Significance to Biomedical Research and the Program of the Institute:

Our Electron Spin Resonance development program is specifically oriented toward developing instrumentation and techniques to solve important biomedical problems. Electron Spin Resonance can yield critical information which cannot be obtained with any other available techniques.

Important groups of applications range from the study of respiratory electron transport in normal and damaged cells, as in myocardial ischimia, to studying the cause of carcinogenesis, to determining the mechanism of action of anticancer drugs, to the study of hematopoesis, to the study of drug metabolism and the P450 enzyme system, to the study of transition metal metabolism and toxicity, to the study of cellular receptors proteins and membrane fluidity, to even the study of alterations in cells due to aging.

Over the past year this program has led to the solution of two important biomedical problems. A mechanism has been determined which can explain the origin of the adriamycin induced cardiomyopathy, which is the third most common cardiomyopathy in the United States (Zweier, J. Biol. Chem., 1984, in press). This work may also provide important information regarding the tumoricidal mechanism of adriamycin.

The distance between transferrin's two iron binding sites was measured and this provides insight into the mechanism of iron transport into all cells of the body (Zweier, 1983, J. Biol Chem. 258).

Proposed Course:

Over the next year we plan to continue work on approaches to increase the sensitivity and develop cavities and resonators for biological problems. The x-band spectrometer will be interfaced with a computer system for data analysis, signal averaging and simulation of acquired spectra. Work will will continue on improving loop gap resonation design. A cell culture system is being developed in order to perform Electron Spin Resonance studies on perfused cultured cells.

Publications:

1. Zweier, J. L.: Electron paramagnetic resonance measurement of the distance between the metal binding sites of transferrin. J. Biol Chemistry 258: 13759-13760, 1983.

2. Zweier, J. L.: Reduction of oxygen by iron-adriamycin. J. Biol. Chemistry, in press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01452-01 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders)*

Time Resolved Fluorescence Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	J. R. Knutson	Sr. Staff Fellow	LTD:NHLBI
Others:	R. Chen	Sr. Investigator	LTD:NHLBI
	L. R. Tamura	Biological Aid	LTD:NHLBI

COOPERATING UNITS *(if any)*

Biology Dept., Johns Hopkins Univ. (L. Brand, L. Davenport, J.M. Beechem, D. G. Walbridge), Georgetown University (P. Hensley), and NHLBI:CM (R. Kincaid).

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	.75	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

A new time-resolved fluorescence spectrometer was designed to incorporate recent advances in laser technology. When completed, this facility will accurately determine very complex fluorescence decay kinetics arising from protein and membrane fluorophores. The rapid acquisition of lifetime data during transient (eg., stopped-flow) events is also tenable with this design.

Specialized, powerful data analysis programs are needed to extract complex decay parameters from fluorescing systems. Thus new global analysis schemes were developed (in collaboration with Dr. Brand's lab) for this task. The combination of this state-of-the-art software with our new instrument should yield the highest level of information available from a fluorescence experiment.

Preliminary studies of lifetime changes linked to protein conformational changes were conducted in collaboration with Dr. Kincaid (dansyl-calmodulin +/-CA^{++}) and Dr. Hensley (OTCase +/-PALO).

1066

Project Description:

Objectives:

We wish to develop instrumental approaches to sharpen and clarify the time resolution of fluorescent signals from proteins and membrane structures. This will help us to untangle the complex emissions that biological systems display. Once these systems are broken down into components, it becomes possible to associate spectral features with reporting groups that represent particular sites and/or conformations. Binding events (and related biological events) can thereafter be examined as they perturb associated spectra. Thus, one can trace the dynamic effects of each event to specific sites in the macromolecule. Fluorescence spectra and decays are exquisitely sensitive indicators of structure and dynamics. The only bar to their effective use is the difficult task of "untangling" complex, mixed signals. The state-of-the-art instrument we are constructing will, when combined with our recent data analysis schemes, greatly facilitate the recovery of multiple spectra from complex systems. These resolved components will help us understand the basic function of dynamic structures in proteins and lipid bilayer membranes.

Major Findings:

The new instrument is still under construction; thus, we can only report findings from those preliminary studies we have done with our modified Ortec system. In addition, some of the data analysis developments were recently presented at meetings and deserve mention.

The new analysis techniques were developed in collaboration with workers at the Johns Hopkins University. These methods combine several types of decay data into the analysis of an overall physical model for the system being studied. This makes the acquisition of spectra connected to various decays DAS = decay associated spectra", Knutson et al, 1982) particularly straightforward, and improves the recovery of hydrodynamic data (Beechem, Knutson and Brand, 1984,a,b).

Preliminary studies of fluorescence decays that change with conformation of proteins were conducted. Dansyl-calmodulin (DCAM) from the laboratory of R. Kincaid (NHLBI, Laboratory of Cellular Metabolism) was examined for calcium dependent changes in fluorescence decay. A change in the mixture of two lifetimes was seen without changes in the lifetimes themselves. This suggests a two-state equilibrium of probe environments (or two distinct sites, one of which acts as reporter) that can be used to monitor active vs. inactive states of the protein. More complete anisotropy and spectral studies are planned for the near future. Control studies with dansyl amino acids have already indicated homogeneous decay, lending further support to the multisite model.

The enzyme Ornithine transcarbamoylase (OTCase) from the laboratory of Preston Hensley (Georgetown University) was examined for tryptophan emission lifetime changes that accompany a known conformational shift (on binding of substrate analogs). A significant dynamic quench (reduction in mean lifetime) was evinced. The multiple subunit origins of this quench will be examined as soon as our scanning capability for the new instrument is in place.

pH-sensitive fluorophores (From W. Hagins,NIADDK:LCP) were examined for their spectral shifts and intrinsic spectral heterogenieity, using a modified Aminco-Bowman spectrofluorometer that Ms. Tamura has interfaced to an HP-87 microcomputer. This arrangement, when combined with her programs, facilitates collection, averaging, smoothing, and correction of fluorescent spectra for detector nonideality. An unexpected dual fluorescence of carboxyfluorescein, a dye commonly used to examine microscopic pH, was found. This dualitly was confirmed with decay measurements

Significance to Biomedical Research and the Program of the Institute:

A casual survey of biomedical literature will rapidly impress a reader with the popularity of fluorescence techniques in Biochemistry. This is largely due to the exceptional sensitivity to surroundings that fluorophores exhibit, along with the variety of signals made available by these probes. Improved fluorescence technology translates directly into improved knowledge of protein and membrane function. Currently, the major limitation to biophysical fluorometry is complexity, and this complexity can be solved with improved instruments and their accompanying data analysis methods.

Proposed Course:

We will construct and test a time-resolved fluorometer with the goal of providing the utmost attainable accuracy and power. We will evaluate model systems including multisubunit protein complexes and bilayer membranes that contain multiple domains. These studies will be conducted using the most recent advances in luminescence data analysis. In all, we will seek to produce the premier fluorescence lifetime and anisotropy instrument, a tool that will reveal sharper detail about macromolecular structure and function.

Publications:

Simultaneous Analysis of Multiple Fluorescence Emission Anisotropy Decays. J. M. Beechem, J. R. Knutson and L. Brand, Biophys. J., Feb. 14, 1984 (Abstract).

Global Analysis of Fluorescence Emission and Anisotropy Decay: Associative and Non-Associative Modeling, J. M. Beechem, J. R. Knutson, and L. Brand, Proc. Am. Soc. Photobiol., July 6, 1984 (Abstract).

Reference:

Knutson, J. R., Walbridge, D. G., and Brand, L.: Decay Associated Spectra Biochem. 21: 4671, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01453-01 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Exploration of Spectroscopic Sources for Ultra Microanalysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. L. Bowman Chief, LTD LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Quantification of metals in nanoliter samples of biological fluids is still limited by problems of source excitation for sufficient time to make a measurement. The helium glow apparatus is the current available method. Previous experience with sealed helium samples tubes has indicated a potential for improvement if smaller tubes could be excited. 12 cm microwave excitation previously used requires a relatively large tube and microwave cavity - 3 cm microwave cavities are considered excited by the newly available Gunn Diodes that may be able to supply sufficient power to excite the smaller tubes if a more efficient cavity can be designed.

A second source the vortex flame has been designed and tested for a micro size source that retains the analytic in the flames for a longer time than conventional flames and is being investigated as a means of exciting the very small sample.

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Project Description:

Objectives:

To explore methods of stimulating the emission of spectral lines for spectroscopic analysis.

Methods:

Two methods have been considered for improvement of spectroscopic sources. One based on a vortex flame and another the excitation by very high frequency microwaves in the 3 cm band. The unique properties of the vortex flame were discovered when it was created as an approach to the construction of quartz micropipettes. The idea being rotate the flame instead of rotating the quartz to get uniform heating around the quartz 1 mm tubes. The system works but vortex forces distort the drawn microsection into a corkscrew shape so that another burner was designed to successfully cope with the quartz pipette problem, the subject of another report.

The spectroscopic properties of the burner were examined by providing an aerosol of the analyte to be admitted to the suction port of the burner and the emission observed visually with a spectrometer. The vortex is created by admitting high pressure oxygen tangential to the larger of two holes of different sizes concentrically bored in a brass cylinder the large hole of about 3 to 4 mm, the smaller 1 to 2 mm and the whole cylinder only 2 cm long. The tangential jet produces a very fast rotating vortex at the junction of the smaller hole so that the central portion becomes a low pressure region that aspirates from the small hole and discharges form the large end. Gas supplied to the aspiration end by another hole at right angles provides fuel to make a very hot region at the center of the vortex where the gas oxygen mixing occurs. The higher pressure oxygen occupies the space against the wall of the burner while the gas air flame becomes a luminous tube inside the vortex without contacting the wall. When only a small amount of gas is admitted the end of the small hole in the cylinder continues to aspirate air, and can be used to admit analyte and buffer gases. The very high velocity vortex causes the analyte to be recirculated in the burning surface to maximize desolvation and multiple emission events. So far only end on observation has been made and several configuration of vortex jets have been explored but multijet systems seem to have little advantage over a very simple set of drilled holes.

The microwave source for spectroscopic excitation of a glow discharge in a helium atmosphere in sealed tubes is an attractive way of exciting ultramicro samples. Sources developed in this lab previously operated in the 12 cm band and used tubes a centimeter long and 1-2 mm i.d. operating at several watts. The present experiments seek to excite much smaller volumes in the 3 cm band at 0.1 watts of power. As micro sources are still

a major technical problem for ultra micro samples. Several 3 cm Gunn diodes were obtained to explore this. Gunn diodes produce 3 cm microwaves directly from a simple DC circuit and feed a 3 cm wave guide. These devices have recently become available and promise to produce enough power to excite micro sources when coupled to a suitable resonator. Present experiments are concerned with the coupling and resonance devices.

Major Findings:

The vortex flame has been tested in several configurations that indicate that gas mixtures and flames can be varied to produce effective sources but so far no specific analytical problems have been attached. The microwave sources have been tested in simple wave guide resonators but so far only neon mixtures have been excited at the watt level. Several diodes feeding a single resonator may be the simple way to get the field strength up to levels needed to excite helium at atmospheric pressure. An unusual finding is the strong odor of ozone produced by the vortex flame presumably created by ultraviolet emission of the intense flame converting the O_2 to O_3. This should be confirmed by spectral observations.

Significance to Biomedical Research and the Program of the Institute:

Spectroscopic sources for exciting ultramicro quantities of ions are widely used in physiologcal research and are generally the main source of error. Improvements would facilitate physiological research.

Proposed Course:

Further experiments will establish operating conditions for the vortex flame. Additional gases will be introduced to provide metastable states to excite analtye ions more efficiently. The Gunn oscillation and wave guide resonator will be built and tested.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01454-01 LTD

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Explosive Disintegration of Intravasacular Atherosclerotic Plaque

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. L. Bowman Chief, LTD LTD:NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

On the basis that laser disintegration of atherosclerotic plaque acts by simply concentrating enough energy in a small spot to vaporize the plaque it is concievable that microexplosion could provide the energy with much simpler and possibly more control than the laser.

An oxyhydrogen shock wave propagated down a conventional angiographic catheter provides a pulse at the catheter tip followed by aspiration of the products into the catheter as the oxyhydrogen products condense to form a partial vacuum. This pulse is probably not energetic enough to destroy plaque but can be used to initiate an explosion of silver azide that can be planted directly into the lesion on the end of a wire that can be visualized radiographically.

Silver azide has been used to destroy bladder stones without undue hazard in patients so the intravascular application may also be feasible.

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Project Description:

Objectives:

To find a means of removing atherosclerotic plaque via transcutaneous intravascular catheters.

Methods:

Some success in the disintegration of plaque by intravascular laser beams has been reported and it appears that if sufficient energy can be delivered to a small volume plaque is vaporized to microscopic particles that can pass through the capillary bed supplied by the vessel treated. We are examining the possibility of delivering the energy to disperse the plaque by the use of microexplosives. Consultation with explosive experts have confirmed that very high energy concentration can be obtained in very small volumes. Also they have had considerable success in dispersing renal and bladder stones with silver azide explosions. A pellet of a few tenths of a milligram exploded inside the patient produces no problems or toxic products.

There are some considerations that make this approach attractive for plaque destruction. The azide can be delivered at the tip of a wire introduced into the catheter and placed radiographically in the plaque. There is also the possibility of shaping the end of the wire to form a focussed shock wave to direct the force. The explosion can be initiated by sending a shock front down the catheter in a stoichiomatic $O_2H_2 + O_2$ mixture down the catheter. When this explosion initiates the azide condensed steam in the catheter it creates a vacuum to suck the product into the catheter.

We have delivered a hydrogen oxygen mixture from electrolysis of water to a #5 angiographic catheter and caused an explosive shock wave to be propagated from the external part of the catheter down to the tip where it produces a minor destructive effect on foam plastic. This impulse however will initiate the silver azide explosion.

Major Findings:

It is too early to evaluate the methods as only the oxyhydrogen valving and spark initiation portion has been completed. The explosive pulse of the oxyhydrogen is propagated thru the catheter without significant pulse palpable but the end of the catheter directed against polystyrene foam produces a dent in the material and could have some use by itself.

Significance to Biomedical Research and the Program of the Institute:

Removal of plaque from vital areas is an established therapeutic goal and this approach may be competitive with the more expensive laser approach currently being tested.

Proposed Course:

Silver and copper azide initiated with oxyhydrogen via a catheter will be tested for the effects on plaque in animal vessels and if promising tested for tolerance by animal experiments.

Publications:

None

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1983 through September 30, 1984

Populations of relatively undifferentiated NG108-15 neuroblastoma-glioma hybrid cells or NS20-Y neuroblastoma cells can be shifted a more differentiated state by increasing the levels of cellular cAMP for several days. In previous studies the differentiated cells were shown to possess functional voltage sensitive channels for Na^+, K^+, and Ca^{2+}, a Ca^{2+}-dependent K^+ channel that is not voltage-sensitive, long neurites, and small clear vesicles and large dense-core vesicles; whereas, these components were absent or were reduced in undifferentiated cells. Differentiated cells also exhibit higher specific activities of choline acetyltransferase and acetylcholinesterase, secrete more acetylcholine when stimulated, and form more synapses with striated muscle cells than do undifferentiated cells.

During the past year, poly A^+ RNA was obtained from differentiated NG108-15 and NS20-Y cells, cDNA was synthesized and then cloned using plasmid pBR322 as the vector. cDNA corresponding to species of poly A^+ RNA that are more abundant in differentiated cells than in undifferentiated cells were purified by repetitive hybridization with poly A^+ RNA from undifferentiated cells; single-stranded nucleic acids then were separated from double-stranded nucleic acids. The species of cDNA that increase in abundance as cells differentiate were cloned. Some clones will be used as probes to study the mechanisms of cAMP-dependent regulation of mRNA in neuroblastoma cells.

Prolonged elevation of cellular cAMP results in an increase in at least one species of protein that is part of the voltage-sensitive calcium channel complex. Concomittantly, cells acquire functional voltage-sensitive calcium channels. Voltage-sensitive calcium channel proteins were purified extensively. The cDNA libraries that have been constructed will be screened for clones that correspond to the channel proteins.

Our previous analysis of glycoproteins synthesized by NG108-15 cells grown in the presence of PGE_1, an activator of adenylate cyclase, or in the absence of PGE_1, was extended during the past year. The cells were incubated with [^{35}S]-methionine solubilized, and the glycoproteins fractionated by wheat germ agglutinin-, ricin-, or lentil lectin-affinity column chromatography and then by 2-dimensional polyacrylamide gel electrophoresis. ^{35}S-Glycoproteins detected by autoradiography were compared with those detected by silver staining. Both methods of analysis showed that elevation of intracellular cAMP levels for several days results in the expression of new glycoproteins, the disappearance of others, changes in the apparent abundance of some glycoproteins, and shifts in the pI of some glycoproteins. However, silver staining revealed many glycoproteins that were not detected by autoradiography, including additional glycoproteins that were expressed only by PGE_1-treated cells. These results suggest that elevation of cAMP levels of NG108-15 cells for several days affects the expression of genes for some glycoproteins and alters the post-translational modification of other glycoproteins. Current studies focus on the purification of sufficient amounts of the regulated

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glycoproteins to obtain partial amino acid sequences for the proteins and to obtain antibodies that recognize the proteins. The cDNA libraries that have been generated will be screened for cDNA that corresponds to some regulated species of glycoproteins.

Whether a peripheral neuroblast will give rise to sympathetic or parasympathetic neurons during differentiation is determined by mechanisms that regulate the expression of the genes for tyrosine hydroxylase and choline acetyltransferase, respectively. An extracellular protein and calcium ions are known to be involved in the regulation of these genes, but the mechanisms of regulation are unknown. Previously, choline acetyltransferase from rat brain was purified to essential homogeneity and 4 monoclonal antibodies that recognize the enzyme were obtained. A large NG108-15 cDNA library was constructed using the bacteriophage expression vector, λgt11. Possible cDNA clones that direct the synthesis of choline acetyltransferase in E. coli have been detected, but further work is needed to establish the identity of the clones.

A monoclonal antibody was obtained previously that recognizes a large dorsal-ventral concentration gradient of a protein in plasma membranes of chicken retina cells. The amount of protein detected is a function of the position of the cells in retina with respect to the dorsal-ventral axis of the retina. The protein is synthesized by proliferating neuroblasts and by nondividing neurons and the the gradient is formed as the retina is formed. The protein was detected on all cells examined in dorsal and middle retina. Cells that were dissociated from retina and cultured in vitro express the amount of gradient protein that would be expected of cells in the intact retina depending upon the original position of the cells in the retina. These results suggest that the gradient is established by an irreversible, clonally inherited mechanism and thereafter, the gradient is perpetuated independently by each cell.

Monoclonal antibody that recognizes the gradient protein, or hybridoma cells synthesizing the antibody, were injected into the amniotic cavity of chick embryos in ovo from the second to the fifth day after fertilization and into the vitreal space of chick embryo eyes to determine whether the antibody affects the development or the spatial organization of the retina. The retinas of embryos were continuously exposed to antibody throughout development from the second to the twentieth day after fertilization. Injection of antibody to the gradient protein into the eye resulted in a marked reduction of synapses and neurites in the inner synaptic layer of the retina; whereas, antibodies synthesized by parental P3X63 Ag8 myeloma cells had no effect.

RNA was isolated from 14 day chick embryo retinas and a large cDNA library was constructed in λgt11 that can be used to direct the synthesis of proteins specified by the cDNA in E. coli. The library currently is being screened for recombinants that direct the synthesis of the gradient protein. Injection of poly A^+ RNA from retina into Xenopus laevis oocytes resulted in the synthesis of the gradient protein. This assay can be used for the purification of mRNA for the gradient protein. The cDNA library also is being screened for transducin subunits in collaboration with A. Spiegel.

Seventy-six hybridoma cell lines were generated that synthesize monoclonal antibodies that bind to 8 day chick embryo optic tectum. Fifteen hundred hybridoma lines were generated from spleen cells of mice immunized with the cervical-thoracic spinal cord and dorsal root ganglia of 8 day chick embryos. Some of the hybridoma lines synthesize antibodies that recognize antigens that are restricted to fiber tracts or neuronal cell body regions of the spinal cord.

Additional information was obtained about other antigens that are recognized by monoclonal antibodies. For example, antigen 13H9 was shown to be a protein with an approximate M_r of 180,000. The antigen is associated with cell membranes of all chick retina cells but has not been detected on neurons or glia in other parts of the nervous system. The antigen defines a functional set of cells in the nervous system.

18B8 antigens are first expressed by ganglion neurons and then by other types of neurons in retina. The antigens are found on cell soma initially, but later in development antigens disappear from cell soma and can be seen in a highly stratified, multi-laminar pattern in the inner synaptic layer of the retina and in a circular "organelle" in the outer synaptic layer. The antigens are expressed by approximately 10% of the cells in retina. In collaboration with Victor Ginsburg and his colleagues, the antigens were shown to be novel gangliosides of unknown structure that contain disialyl residues whose abundance and structure change during development; the location of the gangliosides in retina also changes during retinal development. Most of the antigens are associated with the inner and outer synaptic layers of retina in late embryo and adult retina. In addition, the antigens for many other monoclonal antibodies were characterized and in some cases were partially purified.

A heat-stable, acidic, soluble, bovine brain protein was found that induces neurite outgrowth from chick embryo cerebral cortical neurons at nM concentrations in defined medium. The Neurite Extension Factor (NEF) rapidly stimulates the phosphorylation of a protein with an apparent M_r of 90,000 in the absence of calcium ions or cyclic nucleotides. Phosphopeptide mapping results show that the 90,000 M_r protein is related to an 87,000 M_r protein that is a major substrate for C kinase in brain.

Further information has been obtained on the aggregation of nicotinic acetylcholine receptors on cultured myotubes induced by neuronal factors. In experiments using image intensification to directly observe changes in receptor distribution, and electron microscopy to study changes in the subsurface cytoskeleton and extra-cellular matrix, we have demonstrated discrete steps in the assembly of receptor aggregates from diffuse receptors. The transition from microaggregates of acetylcholine receptors, which appear first, to large dense aggregates is dependent on temperature and involves an increase in the stability of the aggregate.

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The fine structure was studied of regions of myotubes containing microaggregates of nicotinic acetylcholine receptors that form within 90 minutes of exposure to embryonic brain extract. A mixture of rhodamine conjugated α-bungarotoxin and peroxidase conjugated toxin was used so that the formation of receptor microaggregates could be observed directly and so that the distribution of acetylcholine receptors on myotube membranes also could be determined. Small receptor aggregates are converted to larger aggregates that are more stable than the smaller ones. Microaggregates form in the presence of embryonic brain extract and accumulate at temperatures between 18° and 23° but do not form larger aggregates. At 36° C aggregates rapidly form from microaggregates. Microaggregates are destabilized rapidly when brain extract is removed or when sodium azide is added to inhibit ATP formation. In contrast, aggregates remain stable for several hours under these conditions. Azide reversibly blocks the formation of both microaggregates and aggregates at 36° C. Electron micrographs of microaggregates reveal characteristic mounds in the cell surface, subtended by loosely organized cytoplasmic filaments. The receptor aggregates have, in addition, an increased association with basal lamina and a characteristic dense filamentous structure below the cell membrane.

The regulation of the gene coding for preproenkephalin, the precursor of the opioid peptides methionine-and leucine-enkephalin, was investigated. cDNA was cloned in the Pst I site of pBR322. A full-length cDNA clone corresponding to rat striatum preproenkephalin mRNA was found and sequenced. The primary structure of the rat preproenkephalin protein deduced from the nucleotide sequence of the cDNA (269 amino acid residues, M_r 30932) is similar to bovine and human preproenkephalin (78% and 82% matched amino acid residues, respectively) and contains 4 copies of Met-enkephalin, 1 of Leu-enkephalin, 1 of Met-enkephalin-Arg-Gly-Leu, and 1 of Met-enkephalin-Arg-Phe. Southern blot analysis of rat genomic DNA with a probe prepared from the rat preproenkephalin clone suggests that the rat contains a single gene for preproenkephalin. The relative abundances of rat preproenkephalin mRNA are as follows: striatum 100, hypothalamus 11.2, pons + medulla 10.8, spinal cord 10.3, cerebellum 6.1, midbrain 5.9, frontal cortex 4.6, hippocampus 2.0, thalamus 1.6. Electroconvulsive shock treatment (1 sec per day) of rats for 10 days elicited increases of 78% and 0-14% in the relative abundances of preproenkephalin mRNA of the hypothalamus and striatum, respectively.

Preproenkephalin mRNA was detected in NG108-15 mouse neuroblastoma x rat glioma poly A^+ RNA by Northern blot hybridization. The abundance was 1/650 of that of the rat striatum and was increased 3.5 fold by treatment of the cells with the glucocorticoid hormone, dexamethasone, for 4 days. This cell line can be used for the study of preproenkephalin gene expression.

Large cDNA libraries were prepared from cat dorsal root ganglion poly A^+ RNA and rat spinal cord poly A^+ RNA, for the isolation of clones containing cDNA for the precursors of tachykinin neuropeptides.

Histidyl-proline diketopiperazine (cyclo(His-Pro)), a metabolite of the thyrotropin releasing hormone, has been reported to inhibit prolactin secretion from the pituitary, elicit anti-depressant effects, alter cyclic nucleotide

levels, and alter body temperatures. A search for receptors for cyclo(His-Pro) revealed specific binding sites for cyclo(His-Pro) in particulate fractions derived from bovine adrenal cortex or liver, but not in fractions derived from brain or pituitary. A single class of binding sites was found with a K_d of 900 nM and a maximum number of sites of 92 pmol per mg protein. The binding was stereospecific and the histidine moiety of the peptide was a major determinant of the binding. The binding sites for cyclo(His-Pro) were inactivated by incubation of particulate fractions with trypsin or at 100° C. No metabolism of cyclo(His-Pro) was detected.

Some strains of E. coli accumulate toxic levels of methylglyoxal that inhibit cell growth. One such strain was isolated and shown to synthesize a mutant form of the cAMP receptor protein and to lack the gene for adenylate cyclase. Growth of the cells on glucose-6-phosphate, but not glucose, resulted in premature growth arrest due to the accumulation of methylglyoxal. The specific activity of phosphofructokinase in the mutant cells was elevated. The mechanism of growth arrest in the mutant cells was suggested to involve an increase in the synthesis of triose phosphate via glycolysis with spillover of metabolites into a pathway leading to the formation of methylglyoxal.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00009-10 LBG

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Recognition and Synapse Formation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marshall Nirenberg, Chief, LBG, NHLBI
David Trisler, Staff Fellow, LBG, NHLBI
Dana Hilt, Staff Fellow, LBG, NHLBI
Maria Giovanni, Staff Fellow, LBG, NHLBI
Hemin Chin, Guest Worker, LBG, NHLBI
Karl Krueger, Staff Fellow, LBG, NHLBI
Patricia Bray, Biologist, LBG, NHLBI
Hsi-Ping Li, Fogarty Fellow, LBG, NHLBI
Gerald Grunwald, Staff Fellow, LBG, NHLBI

COOPERATING UNITS *(if any)*

William Strauss, Staff Fellow, LDN, NICHD
Victor Ginsberg, Biochemist, LBP, NIADDK

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	10	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Undifferentiated NG108-15 neuroblastoma-glioma hybrid cells or NS20-Y neuroblastoma cells can be shifted to a differentiated state by increasing levels of cellular cAMP for several days. cDNA libraries were constructed for species of mRNA that increase in abundance during differentiation. Elevation of cAMP levels of NG108-15 cells for several days results in the expression of new glycoproteins, the disappearance of others, changes in the apparent abundance of some glycoproteins and shifts in the pI of some glycoproteins. The abundance of a voltage-sensitive calcium channel glycoprotein increases during differentiation. A large NG108-15 cDNA library was constructed in λgt11, for use in cloning cDNA for choline acetyltransferase. A monoclonal antibody that recognizes a plasma membrane protein in chick retina cells that is distributed in a dorsal-ventral gradient in retina was injected into chick embryos in ovo. A marked reduction in synapses and neurites in the inner synaptic layer of the retina was observed. cDNA was synthesized from 14 day chick embryo retina RNA and cloned in λgt11 for use in cloning cDNA for the gradient protein. Many monoclonal antibodies were obtained that recognize antigens in the developing nervous system and some of the antigens were characterized. Several antibodies recognize novel gangliosides. A protein was purified from bovine brain that induces neurite outgrowth at nM concentrations and stimulates the phosphorylation of proteins in the absence of Ca^{2+} or cAMP.

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Project Description

Objectives

To identify and characterize molecules involved in synapse formation and function and to elucidate mechanisms that regulate gene expression for proteins that are required for synapses.

Major Findings

Populations of relatively undifferentiated NG108-15 neuroblastoma-glioma hybrid cells or NS20-Y neuroblastoma cells can be shifted a more differentiated state by increasing the levels of cellular cAMP for several days. In previous studies the differentiated cells were shown to possess functional voltage sensitive channels for Na^+, K^+, and Ca^{2+}, a Ca^{2+}-dependent K^+ channel that is not voltage-sensitive, long neurites, and small clear vesicles and large dense-core vesicles; whereas, these components were absent or were reduced in undifferentiated cells. Differentiated cells also exhibit higher specific activities of choline acetyltransferase and acetylcholinesterase, secrete more acetylcholine when stimulated, and form more synapses with striated muscle cells than do undifferentiated cells.

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Significance to Biomedical Research

New information was obtained concerning synaptogenesis and synaptic functions.

Publications

1. Nirenberg, M., Wilson, S. P., Higashida, H., Rotter, A., Kreuger, K., Busis, N., Ray, R., Kenimer, J., Adler, M., and Fukui, H.: Synapse Formation by Neuroblastoma Hybrid Cells. In: Molecular Neurobiology. Cold Spring Harbor Symposia on Quantitative Biology XLVIII: 707-715. (1983).

2. Nirenberg, M., S. Wilson, H. Higashida, A. Rotter, K. Krueger, N. Busis, R. Ray, J. G. Kenimer, and M. Adler.: Modulation of Synapse Formation by Cyclic Adenosine Monophosphate. Science 222: 794-799. (1983).

3. de Blas, A., Adler, M., Shih, M., Chiang, P. K., Cantoni, G. L., and Nirenberg, M.: Inhibitors of CDP-choline synthesis, action potential calcium channels, and stimulus-secretion coupling. Proc. Natl. Acad. Sci. 81: 4353-4357. (1984).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

DESCRIPTIVE INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL00017-09 LBG

October 1, 1983 - September 30, 1984

Acetylcholine Receptors

Mathew P. Daniels, Research Biologist, LBG-NHLBI
Anthony J. Olek, Staff Fellow, LBG-NHLBI
Jacqueline G. Krikorian, Guest Worker, LBG-NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	3	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Our aim is to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. Our recent work has focused upon the factors, extinsic and intrinsic to the developing skeletal muscle fiber, which regulate the distribution of nicotinic acetylcholine receptors. Acetylcholine receptor aggregation is induced on cultured myotubes by neuronal factors, and this system is used to study the mechanisms of receptor aggregation, as well as the stabilization or elimination of aggregates which occur in developing neuromuscular junctions.

In experiments using image intensification to directly observe changes in receptor distribution, and electron microscopy to study changes in the subsurface cytoskeleton and extracellular matrix, we have demonstrated discrete steps in the assembly of receptor aggregates from diffuse receptors. The transition from the clouds of microaggregates, which first appear, to large, dense aggregates is temperature dependent and involves an increase in stability, as seen when the aggregation factor is removed or sodium azide is added to reduce ATP formation. This transition is correlated with the appearance of specific surface structures. In microaggregates, there are characteristic mounds in the cell surface, subtended by loosely organized cytoplasmic filaments. The aggregates have, in addition, an increased association with basal lamina, and a characteristic dense filamentous structure below the cell membrane. Factor-induced receptor aggregates develop optimally at 36°, are rapidly but reversibly destabilized at 38°, and are more stable at 24°.

Project description:

Objectives:

Our aim has been to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. Our recent work has focused on factors, extrinsic and intrinsic to the developing muscle fiber, which regulate the distribution of nicotinic acetylcholine (ACh) receptors. In particular, we have been investigating the induction of ACh receptor aggregation on muscle cells in culture, by soluble macromolecules from neurons. This system serves as a model in which to study the mechanisms of the aggregation of ACh receptors which occurs at the developing neuromuscular synapse, and the mechanisms of stabilization or elimination of receptor aggregates as synapses mature, are remodeled, or are eliminated.

Methods:

We have stained rat skeletal myotubes grown in monolayer culture with rhodamine-labeled α-bungarotoxin (αBT) in order to visualize ACh receptor sites with the fluorescence microscope. Sequential observations of ACh receptor distribution are made by use of a video image intensification system.

The distribution of basement membrane proteins in the cultures is determined by indirect immunofluorescence, using antisera against purified proteins. We are using acrylamide gel electrophoresis in combination with immunoblotting and immunoprecipitation to confirm the specificities of the antisera relative to the muscle cell cultures.

Alpha-bungarotoxin coupled to horseradish peroxidase is used to examine the distribution of ACh receptors on myotube surfaces at the electron microscopic level.

ACh receptor aggregating material is prepared from extracts of fetal pig brain.

Major Findings:

Recent Background

We have previously shown that: a) ACh receptor aggregates form maximally in 4-6 hours of the exposure of myotubes to embryonic brain extract. b) The formation of these large, dense aggregates is preceded by the appearance of clouds of microaggregates (less than 1 micron in diameter). c) The dense aggregates formed within 4-6 hours have specializations of the extracellular matrix and subsurface cytoskeleton similar in several aspects to those found at the neuromuscular junction.

In the past year we have made advances in the following areas:

1. Fine structural changes associated with the formation of ACh receptor microaggregate clouds: We have studied the fine structure of regions of myotubes containing microaggregate clouds which have formed within 90 minutes of exposure to embryonic brain exract. Use of a mixture of

rhodamine conjugated α-bungarotoxin and peroxidase conjugated toxin allows us to observe directly the formation of the microaggregate clouds, and then to detect the distribution of ACh receptors on the electron microscopic level. The microaggregates resemble aggregates in two ways: They show changes in membrane contour consisting of distinctive mounds or ridges in the cell surface, and they contain a subsurface region occupied by fine filaments which appear to exclude most other organelles. However, the microaggregates show relatively little of the basal lamina and dense filamentous submembrane specialization found in the aggregates. Thus, we are now able to describe steps in the morphological assembly of the ACh receptor aggregate and to tentatively relate changes in the properties of the forming aggregates (below) to the appearance of specific structural components.

2. Stages in the formation of ACh receptor aggregates: We have now obtained physiological evidence that the microaggregate cloud is the precursor of the receptor aggregate, but is less stable than the aggregate. Microaggregates will form (in the presence of embryonic brain extract) and accumulate at temperatures between 18 and 23°, but will not become aggregates. At 36°, aggregates will rapidly evolve from the microaggregate clouds. Microaggregates are destabilized rapidly when brain extract is removed, or when sodium azide is added to inhibit ATP formation. In contrast, aggregates remain stable for several hours under these conditions. Azide reversibly blocks the formation of both microaggregates and aggregates at 36°. (In this respect, the mechanism of ACh receptor aggregation appears different from the patching of antigens on cell surfaces caused by bivalent antibodies.). The differences in stability between microaggregate clouds and aggregates may be related to the structural changes described in section 1.

3. The stability and disassembly of ACh receptor aggregates: We have studied the effects of temperature on the ACh receptor aggregates formed after 4 hours exposure to brain extract. Aggregates are more stable (half life greater than 12 hours) at 24° than at 36° (half life about 7 hours) and are destabilized rapidly (half life 1.5-2 hours) at 38°. The effect of temperature elevation is reversible, since aggregates will form again if brain extract is reapplied at 36°.

Significance to Biomedical Research:

An understanding of the control mechanisms involved in the organization of neurotransmitter receptors at the developing synapse is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. Our studies on the interactions between neuronal factors, extracellular matrix components, and cytoskeletal structures may lead to a better understanding of the mechanisms whereby neurons control or modulate the distribution of receptors on muscle fibers and on other neurons, during synapse development and after.

Proposed Course:

1. In a collaborative effort with investigators at the University of Maryland, the molecular composition of the ACh receptor aggregates and associated structures will be studied by immunocytochemistry, first at the light microscopic level, then with the electron microscope. The changes in the distribution of extracellular matrix components during receptor aggregation will be followed directly, using labeled Fab fragments of specific antibodies.

2. The cytoskeletal and extracellular matrix structures associated with developing ACh receptor aggregates will be studied by current preparation techniques which allow better preservation and resolution of their supramolecular architecture and their possible linkage with the receptors. These techniques involve rapid freezing, cryofracture, freeze etching, and surface replication.

3. The mechanisms involved in the disassembly of receptor aggregates will be studied. We will attempt to distinguish between dispersal of the aggregated receptors and their internalization. We will determine whether the structural specializations found in the aggregates are preserved or broken down during aggregate disassembly. We will also determine whether altering the composition of the extracellular matrix, by adding laminin or collagen type IV, or by treating the cells with ascorbate, will alter the stability of the ACh receptor aggregates.

4. In a recently initiated collaboration with investigators at Stanford University, we will attempt to determine whether the ACh receptor aggregating protein in embryonic brain extract is functionally and antigenically similar to the active protein in a more highly purified preparation from the extracellular matrix of the electroplax (a highly enriched analogue of the neuromuscular junction).

Publications:

Busis, N.A., Daniels, M.P., Bauer, H.C., Pudimat, P.A., Sonderegger, P., Schaffner, A.E. and Nirenberg, M.: Three cholinergic neuroblastoma hybrid cell lines that form few synapses on myotubes are deficient in acetylcholine receptor aggregation molecules and large dense-core vesicles. Brain Res. In Press.

Daniels, M.P., Vigny, M., Sonderegger, P., Bauer, H-C. and Vogel, Z.: Association of laminin and other basement membrane components with regions of high acetylcholine receptor density on cultured myotubes. Int. J. Devel. Neuroscience 2: 87-99, 1984.

Olek, A.J., Pudimat, P.A. and Daniels, M.P.: Direct observation of the rapid aggregation of acetylcholine receptors on identified cultured myotubes after exposure to embryonic brain extract. Cell 34: 255-264, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL00018-07 LBG

October 1, 1983 - September 30, 1984

PROJECT TITLE (Type from left margin, do not indent, within the border)

Regulation of the biosynthesis of the opioid peptides and other neuropeptides

PRINCIPAL INVESTIGATOR(S) (List professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

Steven L. Sabol, M.D., Ph.D., Medical Officer (Research), LBG, NHLBI

Kazuaki Yoshikawa, M.D., Ph.D., Visiting Fellow, LBG, NHLBI
Steven F. Lee, B.S., Physical Science Aide, LBG, NHLBI
Christianna Williams, Summer Aide, LBG, NHLBI

COOPERATING UNITS (If any)

Jau-Shyong Hong, Ph.D., Section Chief, Lab. Behav. Neurol. Toxicol, NIEHS, Research Triangle Park, NC

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.6	2.0	0.6 (summer students)

CHECK APPROPRIATE BOX(ES)

(a) Human subjects [ ] (b) Human tissues [X] (c) Neither

(a1) Minors [ ]

(a2) Interviews [ ]

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The regulation of the gene coding for preproenkephalin, the precursor of the opioid peptides methionine- and leucine-enkephalin, is under investigation in several neuronal systems. A full-length cDNA clone was derived by recombinant DNA methodology and sequenced in order to provide an ideal hybridization probe for rat ppEnk mRNA. The deduced amino acid sequence of rat ppEnk (M_r 30932) is similar to those of bovine and human ppEnk with respect to opioid peptide sequences and processing sites. Southern blot analysis of rat genomic DNA with a probe prepared from the rat ppEnk clone is consistent with the existence of a single rat ppEnk gene. The rat ppEnk probe was also found to hybridize in a highly sensitive and specific manner with rat ppEnk mRNA on Northern blots of poly(A)+ or unselected total RNA from various regions of the rat brain. A dot-blot hybridization assay was developed and used to determine the following relative abundances of ppEnk mRNA: striatum 100, hypothalamus 11.2, pons + medulla 10.8, spinal cord 10.3, cerebellum 6.1, midbrain 5.9, frontal cortex 4.6, hippocampus 2.0, thalamus 1.6.

Electroconvulsive shock treatment (1 sec/day) of rats for 10 days elicited increases of 76-79% and 0-14% in the relative abundances of ppEnk mRNA in poly(A)+ RNA of the hypothalamus and striatum, respectively. The ppEnk mRNA increase in the hypothalamus may explain the previously found 65-100% increase in the hypothalamic Met-enkephalin content elicited by electroconvulsive shock.

ppEnk mRNA was detected in the NG108-15 mouse neuroblastoma x rat glioma hybrid cell line by Northern blot hybridization. The abundance was 1/650 of that of the rat striatum and was increased 3.5-fold by treatment of the cells with a glucocorticoid hormone (1 uM dexamethasone) for 4 days. This cell line is a useful homogeneous neuronal system for the study of ppEnk gene expression.

Large cDNA libraries have been prepared from mammalian neuronal mRNA, including cat dorsal root ganglion mRNA, for the isolation of clones containing cDNA for the precursors of tachykinin neuropeptides.

1076

Project Description

Objectives

The pentapeptides Met- and Leu-enkephalin are endogenous opiate-receptor ligands that modulate the release of neurotransmitters in widely distributed neuronal pathways of the central nervous system and gut. We previously identified and characterized the enkephalin precursor gene product preproenkephalin (ppEnk) by cell-free translation of mRNA from bovine and rat enkephalin-containing tissues such as the rat striatum (see 1983 annual report). Our current major objective is to study the regulation of ppEnk gene expression in neuronal systems such as the rat brain and rodent neuroblastoma cell lines. The rat brain is important for such studies since most work on the function of opioid peptides and opiate receptors in the nervous system is being conducted in the rat. Thus, fundamental information about the rat ppEnk mRNA and protein primary structures and the gene sequence and organization are needed. Furthermore, a sensitive and specific hybridization probe for rat ppEnk mRNA is needed.

Several peptides of the tachykinin group have been found in mammalian nervous tissue. In addition to the well-known substance P (SP), the tachykinins substance K (SK) and neuromedin K (NK) have been recently identified. The structure of a bovine common precursor for SP and SK is now known, but the precursor for NK has not been characterized. The goals of our work are to obtain and characterize cDNA clones for the NK precursor as well as clones for the rat and human SP-SK precursor in order to study the regulation of expression of the SP/SK and NK genes in experimental rats and in human disease.

Methods Employed

Messenger RNA purification, cell-free translation, immunoprecipitation/gel electrophoresis, mRNA Northern-blot and dot-blot hybridization, cDNA cloning, colony hybridization with cDNA probes and oligonucleotides, DNA sequencing.

Major Findings

A. Rat brain preproenkephalin mRNA: cDNA cloning, primary structure, and distribution in the central nervous system.

A cDNA clone library of 11,000 transformants was constructed from poly(A)+ RNA of Fischer rat striatum. cDNA inserts were cloned in the Pst I site of the plasmid vector pBR322. The library was screened for inserts coding for rat ppEnk by colony hybridization at reduced stringency with a human ppEnk cDNA probe (gift of Dr. E. Herbert, Univ. of Oregon). The insert of one positive clone, pRPE2, was sequenced and found to contain the coding sequence (810 bases), as well as 316 and 155 bases of the 3' and 5' untranslated regions, respectively, of rat ppEnk mRNA. The deduced primary structure of the rat ppEnk protein (269 amino acids, M_r 30,932) is similar to those of previously sequenced bovine and human ppEnk proteins (78% and 82% matched residues, respectively) and contains 4 copies of Met-enkephalin, one of Leu-enkephalin, one of Met-enkephalin-Arg-Gly-Leu, and one of Met-enkephalin-Arg-Phe. Cell-free translation of rat striatal mRNA selected by hybridization with pRPE2 DNA resulted in the synthesis of a major M_r 31,000 protein that was detected by SDS-polyacrylamide gel electrophoresis and specifically immunoprecipitated by

Met-enkephalin-Arg-Phe antibodies. This observed size is consistent with that deduced from the amino acid sequence.

Fragments of the pRPE2 clone insert were labeled with ^{32}P by nick-translation and used as hybridization probes. On Southern blots of restricted rat genomic DNA, the probes hybridized with a single DNA band for each restriction digest, a result consistent with a single rat ppEnk gene per haploid genome. A ^{32}P-labeled 941-base pair restriction fragment of pRPE2, which includes the entire coding region was used as a sensitive hybridizaiton probe for rat ppEnk mRNA. This probe hybridized specificially with ppEnk mRNA (size 1500 bases) on Northern blots of either poly(A)+ or unselected (total) RNA of all rat brain regions, but not liver. A dot-blot assay was developed to detect with increased sensitivity ppEnk mRNA in small amounts of total RNA from brain regions, including regions having a low abundance of ppEnk mRNA. For total RNA, the relative abundances were as follows: striatum 100, hypothalamus 11.2, pons + medulla 10.8, spinal cord 10.3, cerebellum 6.1, midbrain 5.9, frontal cortex 4.6, hippocampus 2.0, and thalamus 1.6. These relative abundances are consistent with the distribution of enkephalin-containing neuronal cell bodies as determined by immunocytochemistry. While absolute abundances have not been determined, the abundance of ppEnk mRNA in striatal poly(A)+ RNA was estimated to be approximately 0.02% by cell-free translation.

B. Effect of electroconvulsive shock on rat brain preproenkephalin mRNA abundance

Hong et al. showed in 1979 that daily administration of electroconvulsive shock (ECS) (1 sec shock per day) to rats for 10 days increases the Met-enkephalin content in select brain regions, particularly hypothalamus, by 65-100%; other regions had much less or no increase. To determine whether ECS increases the relative abundance of ppENK mRNA in hypothalamus, this mRNA was quantitated by blot hybridization with the rat ppEnk cDNA probe and hy cell-free translation/immunoprecipitation. Analysis by the latter method revealed that ECS elicits increases of 79% and 14% in the ppEnk mRNA activity in the hypothalamus and striatum, respectively, while ECS does not affect the general translational activity of poly(A)+ RNA from these regions. The rat ppEnk probe hybridized with an apparently single species of poly(A)+ RNA of 1450 bases from both regions. Dot-blot hybridiztion of poly(A)+ RNA with this probe indicated that ECS elicits a 76% increase in the ppEnk mRNA abundance in the hypothalamus and no significant change in the striatum. Thus, results obtained by two independent methods indicate that ECS treatment elevates ppEnk mRNA levels in hypothalamic neurons with little effect in striatal neurons.

C. Regulation of preproenkephalin mRNA in clonal cell lines

Low levels (0.1 pmol/mg protein) of Met- and Leu-enkephalins were detected in the NG108-15 mouse neuroblastoma x rat glioma hybrid cell line and N1E115 mouse neuroblastoma cell lines by other laboratories. In the case of NG108-15 cells, the levels were increased by treatment with glucocorticoid hormones such as dexamethasone for 1 day or longer. These cell lines, the only ones known to contain enkephalins, are potentially interesting systems to study transcriptional regulation of the preproenkephalin gene in a homogeneous population of cells. Total RNA prepared from untreated and 1 uM dexamethasone-treated NG108-15 or N1E115 cells were Northern-blotted and probed with a ^{32}P-labeled

fragment of the pRPE2 clone under stringent conditions of hybridization. A band of 1500-base length, the size of ppEnk mRNA, was detected in RNA of NG108-15 cells but not N1E115 cells. In initial experiments the abundance (per ug RNA) in untreated NG108-15 cells was found to be 1/650 of that of rat striatum and was increased 3.5-fold by dexamethasone treatment. We conclude that NG108-15 cells are a feasible system in which to study regulation of ppEnk gene expression by hormones, differentiating agents, etc.

D. Biosynthesis of mammalian tachykinins

Because the dorsal root ganglion (DRG) is relatively rich in cell-bodies of substance P-containing neurons, poly(A)+ RNA was prepared from cat DRG tissue obtained from Dr. O. Viveros (Wellcome Laboratories). Proteins synthesized by cell-free translation of DRG mRNA were immunoprecipitated with internally-directed antibodies against substance P (SP). A protein of M_r 70,000 was immunoprecipitated and was displaceable from antibodies by unlabeled SP. However, during the course of this work, another laboratory reported the cloning and sequencing of cDNA for an SP/SK precursor of M_r 15,000. Thus the immunoprecipitated protein found by us may have a region structurally similar to SP but not necessarily related to SP biosynthesis.

In a potentially more fruitful study, a cat DRG cDNA library of 100,000 clones and a rat spinal cord cDNA library of 600,000 clones have been prepared by recently published methods that result in good yields of full-length double-stranded cDNA and high transformation efficiencies (3-10 x 10^6 transformants per ug cDNA). These libraries are being screened with synthetic heptadecadeoxynucleotides that are complementary to the mRNA sequences of SP and NK, with the goal of isolating clones containing prepro-SP and prepro-NK cDNA.

Significance to Biomedical Research and the Program of the Institute

Enkephalinergic neurons are thought to be important in pain perception, stress, neuroendocrine regulation, regulation of blood pressure and respiration, and possibly behavior. Fundamental knowledge of the regulation of enkephalin biosynthesis at the genetic level is important for understanding the physiological functions of enkephalins. Most physiological and pharmacological studies on the functions of opioid peptides and opiate receptors are being carried out in the rat. The rat ppEnk cDNA clone derived by us is a much more sensitive probe for rat ppEnk mRNA than the previously cloned human and bovine ppEnk cDNAs. Thus, the rat probe should be the ideal probe for quantitating the low amounts of ppEnk mRNA in discrete regions of the rat brain and in rodent cell lines in a wide variety of experimental studies.

The effect of ECS treatment on enkephalin biosyntheis in the brain is potentially relevant to the antidepressant effects of electroconvulsive therapy in man. Hong et al. noted found similarities in the temporal characteristics of the ECS-elicited increase of the enkephalin content in the rat brain and the anti-depressant effect of shock in man, but a causal relationship has not been established.

Studies on the nature of the mammalian tachykinin precursors and their genes would provide a foundation for studies on the regulation of the biosynthesis of these putative neurotransmitter peptides. It is possible that

such studies will demonstrate that the tachykinin family of peptides is as complex and as widely distributed in the nervous system as the opioid peptides.

Proposed course

1. Mapping of ppEnk mRNA concentrations in discrete nuclei and micro-punches of rat brain.
2. Preparation of suitable single-stranded probes for rat ppEnk mRNA for in situ hybridization histochemistry.
3. Continued studies on the regulation by hormones, drugs, and disease of ppEnk mRNA concentrations in brain and cell lines.
4. Characterization of clones containing prepro-SP/SK and prepro-NK cDNA inserts and their utilization for studies on the molecular genetics and regulation of biosynthesis of these precursors.

Publications

1. Yoshikawa, K., Williams, C., and Sabol, S.L.: Rat brain preproenkephalin mRNA: cDNA cloning, primary structure, and distribution in the central nervous system. J. Biol. Chem, in press, 1984.

2. Yoshikawa, K, Hong, J.S., and Sabol, S.L.: Electroconvulsive shock increases preproenkephalin mRNA abundance in the rat hypothalamus (1984). Proc. Natl. Acad. Sci. U.S.A., in press.

3. Hong, J.S., Yoshikawa, K., Kanamatsu, T., and Sabol, S.L.: Modulation of striatal enkephalinergic neurons by antipsychotic drugs. Federation Proceedings, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

OFFICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00151-14 LBG

October 1, 1983 - September 30, 1984

The Biology of Cyclic Nucleotides in E. coli

Alan Peterkofsky, Research Chemist, Chief, Macromolecules Section, LBG, NHLBI, NIH
Prasad Reddy, Senior Staff Fellow, LBG, NHLBI, NIH
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Ellen Liberman, Staff Fellow, LBG, NHLBI, NIH

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Macromolecules Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	3.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Under certain growth conditions, some strains of Escherichia coli accumulate toxic levels of methylglyoxal. One such strain was isolated and shown to synthesize a mutant form of the cyclic AMP receptor protein and to have the gene for adenylate cyclase missing. During growth on glucose-6-phosphate, but not glucose there was premature growth arrest due to accumulation of methylglyoxal. The mechanism of this growth arrest is suggested to involve unbalanced metabolism through the glycolytic pathway with spillover of metabolites into a pathway leading to the formation of methylglyoxal.

1695

Project Description:

Objectives: The metabolisim of carbon sources during growth of Escherichia coli is subject to a variety of controls that guarantee optimal growth. Some mutations may lead to effects on growth control and produce growth inhibition. One type of growth inhibition that has been reported is associated with the accumulation of toxic concentrations of methylglyoxal. We isolated a strain of E. coli that showed this type of growth inhibition and studied the mechanism of this phenomenon.

Methods Employed:

Mutants of E. coli were isolated and grown under standard conditions. Assays for sugar transport and methylglyoxal accumulation were performed. Metabolite levels (glutathione, pyridine nucleotides, cyclic GMP) were measured. A variety of enzyme assays (methylglyoxal synthase, glyoxalase, phosphoglucose isomerase, phosphofructokinase, aldolase, triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase) were carried out.

Major Findings:

When the mutant was grown in medium supplemented with glucose-6-phosphate there was a premature growth inhibition and methylglyoxal accumulated in the medium. The mutant was characterized as an adenylate cyclase deletion strain which, in addition, produced a mutant form of the cyclic AMP receptor protein. A comparison of a variety of cell properties was made between the mutant and a reference strain that did not exhibit growth inhibition. A defect in transport of glucose-6-phosphate or in the levels of the metabolites glutathione, DPN or cyclic GMP was eliminated as a cause of the growth inhibition. A study of the activities of various glycolytic enzymes revealed that the level of phosphofructokinase was elevated in the mutant. Our interpretation of this finding is that growth inhibition in the mutant may be due to increased production of triose phosphate, some of which is converted to methylglyoxal.

Significance to Biomedical Research and the Program of the Institute:

Growth regulation is a central mechanism in biology. It is of great interest to develop an understanding of the various influences on this complex phenomenon.

Proposed Course:

The finding that mutation in the cAMP receptor protein leads to aberrations in growth regulation is of some interest. The probability is that the mechanism underlying the change is at the level of changes in gene expression. It is our intention to continue to study effects of mutations in the cAMP receptor protein on gene expression.

Publications:

1. Puskas, R., Fredd, N., Gazdar, C. and Peterkofsky, A.: Methylglyoxal-mediated Growth Inhibition in an Escherichia coli cAMP Receptor Mutant. Arch. Biochem. Biophys. 223: 503-513, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00152-10 LBG

PERIOD COVERED

October 1, 1983 - September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism of Peptide Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Alan Peterkofsky, Research Chemist, Chief, Macromolecules Section LBG,NHLBI,NIH
Fiorenzo Battaini, Visiting Associate, LBG, NHLBI, NIH
Yitzhak Koch, Visiting Associate, LBG, NHLBI, NIH
Yoshiyuki Takahara, Guest Worker, LBG, NHLBI, NIH

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Macromolecules Section

INSTITUTE AND LOCATION

NIHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Histidyl-proline diketopiperazine (cyclo(His-Pro)), a metabolite of the neuropeptide thyrotropin releasing hormone, has been shown to possess intrinsic biological activities. The binding of this peptide to various tissue particulate preparations was investigated. While the peptide showed no apparent binding to particulate fractions derived from brain, pituitary, and some other tissues, binding to adrenal and liver was demonstrated. The binding of cyclo(His-Pro) to bovine adrenal cortical particles was further characterized. Binding at equilibrium was greater at 4°C than at 37°C. The binding was dependent on tissue concentration, showed a pH optimum between 7 and 8, and was inactivated by treatment of the particulate fraction with trypsin or by boiling. The interaction of cyclo(His-Pro) with the tissue was not associated with any metabolism of the peptide. Kinetic studies of association of cyclo(His-Pro) with adrenal cortical particles indicated a single class of binding sites with a dissociation constant of approximately 900 nM and a maxium number of sites of 92 pmoles/mg protein. The binding was stereospecific and the histidine moiety of the peptide was the major determinant of the binding. A variety of catechols, serotonin and histamine competed with cyclo(His-Pro) for binding with concentrations for 50% displacement ranging from 17-450 uM. Cyclo(His-Pro) did not affect monoamine oxidase or adenylate cyclase activity in adrenal cortical particulate preparations.

1017

Project Description:

Objectives: Histidyl-proline diketopiperazine [cyclo(His-Pro)] has been shown to be produced by metabolism of thyroptropin releasing hormone by brain extracts. This pathway has some physiological significance since the diketopiperazine has been characterized as a bioactive peptide; cyclo(His-Pro) was reported to inhibit prolactin secretion from pituitary, and to elicit antidepressant effects, changes in cyclic nucleotide levels and changes in body temperature through a central action.

The precise mechanism by which any of these effects of cyclo(His-Pro) occurs has not been elucidated. As a first step in that direction, a search for receptors for cyclo(His-Pro) has been initiated. In a previous study the clearance, distribution and binding of cyclo(His-Pro) in the rat was measured. It was found that the peptide was rapidly cleared from the circulation and found unchanged in the urine. It was shown that adrenal and liver were sites of accumulation of radioactive cyclo(His-Pro). Studies of the binding of [^{3}H]cyclo(His-Pro) to particulate preparations derived from adrenal and liver were therefore carried out. In the present study, the nature of the binding of the diketopiperazine to adrenal cortical particles was characterized.

Methods Employed:

Particulate fractions were prepared from homogenates of fresh or frozen bovine adrenal glands. Radioactive cyclo(His-Pro) was incubated with the particulate fraction and then incubation mixtures were deposited on glass fiber filters. The binding of radioactivity to the particulate fraction trapped on the filters was determined.

Major Findings:

Using this methodology, it was possible to demonstrate a specific binding of cyclo(His-Pro) to particulate fractions derived from liver or adrenal. Low temperature promoted binding. The decreased binding at higher temperature was reversible. There was no detectable metabolism of cyclo(His-Pro) associated with the binding. The optimum pH of the binding was between 7 and 8. A study of the capability of a variety of peptides to compete for the binding of cyclo(His-Pro) was carried out. The results demonstrated that the binding of cyclo(His-Pro) was quite specific. A Scatchard analysis indicated that there was one class of binding sites with a Bmax of 92 pmol/mg protein and a K_d of 886 nM.

Significance to Biomedical Research and the Program of the Institute:

Cyclo(His-Pro) has been shown to have a variety of biological activities. An understanding of the mechanism by which these activities are accomplished will help to understand human physiology better.

Proposed Course:

The finding that cyclo(His-Pro) binds specifically to a particulate fraction of adrenal tissue is noteworthy. The nature of the binding site and its possible function have not yet been elucidated. There is no metabolism of cyclo (His-Pro) associated with the binding. Cyclo(His-Pro) does not stimulate adenylate cyclase in adrenal membranes. The endogenous monoamine oxidase of adrenal tissue was not modulated by cyclo(His-Pro). Other studies will be performed in a search for a biological function for the binding of cyclo(His-Pro) to the adrenal particulate fraction.

Publications:

1. Battaini, F., Koch, Y., Takahara, Y. and Peterkofsky, A.: Specific Binding to Adrenal Particulate Fraction of Cyclo(Histidyl-Proline), a TRH Metabolite. Peptides 4:89-96, 1983.

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